UNIVERSIDAD COMPLUTENSE DE MADRID

FACULTAD DE MEDICINA

TESIS DOCTORAL

Estudio del papel en la resistencia a antiangiogénicos en cáncer renal de células claras (CRCC) de los polimorfismos de nucleótido único (SNPs)

MEMORIA PARA OPTAR AL GRADO DE DOCTOR

PRESENTADA POR

Jesús García-Donas Jiménez

Directores

Cristina Rodríguez González de Antona
José Luis González Larriba

Madrid, 2017

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Tesis doctoral que presenta para optar al título de
Doctor por la Universidad Complutense de Madrid
el licenciado en Medicina y Cirugía

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Dr. José Luis González Larriba Jefe de la Unidad de Tumores Torácicos y Genitourinarios del Hospital Universitario Clínico San Carlos , como Profesor Titular de Oncología del Departamento de Medicina de la Facultad de Medicina de la Universidad Complutense de Madrid, como Director,

CERTIFICAN:
Que Don Jesús García-Donas Jiménez, licenciado en Medicina y Cirugía por la Universidad Complutense de Madrid, ha realizado la presente Tesis Doctoral "Estudio del papel en la resistencia a antiangiogénicos en cáncer renal de células claras (CRCC) de los polimorfismos de nucleótido único (SNPs)" y que a su juicio reúne plenamente todos los requisitos necesarios para optar al Grado de Doctor, a cuyos efectos será presentada en la Universidad Complutense de Madrid, autorizando su presentación ante el Tribunal Calificador.

Y para que así conste se extiende el presente certificado,

Madrid, 8 de Octubre 2015

Vª Bª de la Directora

Dra. Cristina Rodríguez González de Antona

Vª Bª del Director

Dr. José Luis González Larriba

La presente Tesis Doctoral se realizó en el Hospital Universitario Fundación Alcorcón (Madrid), el Centro Nacional de Investigaciones Oncológicas (CNIO) de Madrid y el Centro Integral Oncológico Clara Campal de Madrid, entre los años 2007 y 2015 bajo la supervisión de la Dra. Cristina Rodríguez López de Antona y el Dr. José Luis González Larriba

Las siguientes becas, ayudas y proyectos científicos han permitido la realización de esta Tesis Doctoral:

Proyectos de investigación en Salud ISCIII: Proyecto multicéntrico para la identificación de biomarcadores de resistencia a fármacos antiangiogénicos de primera línea en cáncer renal metastásico y su validación clínica prospectiva. Nº expediente PI13/0622:

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Estudio del papel en la Resistencia a antiangiogénicos en cáncer renal de células claras (CRCC) de los polimorfismos de nucleótido único (SNPs)

A Mertxe Alonso,

por darme el empujón final para acabar este proyecto

ABSTRACT

Los polimorfismos de nucleótido único son pequeñas variaciones en la secuencia de nuestros genes que, aun careciendo de significado patológico, son reponsables de parte de las diferencias fenotípicas interindividuales.

Estas variaciones sí pueden implicar cambios en la actividad de las proteínas resultantes que, aunque pasen desapercibidas en la vida cotidiana, desembocarían en importantes diferencias en situaciones de estrés, como por ejemplo la administración de fármacos con una estrecha ventana terapéutica.

Dado que los antiangiogénicos actúan inhibiendo el crecimiento del endotelio vascular, constituido por células sanas carentes de mutaciones, los polimorfismos representan un mecanismo plausible de resistencia a dichos fármacos.

En la presente tesis se recogen los resultados de una intensa línea de investigación desarrollada por nuestro grupo en el campo de los SNPs y la predicción de eficacia y toxicidad en cáncer renal.

Para ello pusimos en marcha en 2007 un estudio observacional prospectivo, denominado SUT-REN-07, en el que recogimos de forma sistemática muestras de sangre periférica para la extracción de ADN germinal y tejido tumoral parafinado de más de 100 pacientes con cáncer renal de células claras metastásico que fueran a recibir sunitinib dentro de la práctica asistencial..

Gracias a dicho estudio hemos podido completar tres grandes trabajos, que constituyen el cuerpo de esta tesis doctoral. Como principal hallazgo, hemos identificado dos SNPs en el gen VEGFR3 asociados a resistencia a sunitinib y un tercero, en el enzima CYP3A5, asociado a un mayor riesgo de reducción de dosis.

También estudiamos los niveles de expresión proteica, determinados por inmunohistoquímica, de diferentes genes relacionados con la hipoxia y la proteína VEGFR3.

Interesantemente, la baja expresión de VEGFR3 se asoció con la presencia de los SNPs de resistencia previamente mencionados.

Por ultimo, conseguimos validar en nuestra serie, los hallazgos realizados por un grupo independiente en los que determinados SNPs en el gen IL-8 condicionaban también resistencia a antiangiogénicos.

SUMMARY

Single Nucleotide Polymorphisms are little changes in DNA sequence that do not make any difference in daily life but can rise as a major conditionators in stress situations scuh as exposition to antiangiogenic drugs.

Since the real target of antiangiogenic treatments are endothelial cells rather than tumor cells, we aimed to study the role of SNPs in resistance to sunitinib.
With that scope we performed a prospective observational study in order to collect biological samples from patients diagnosed of advanced clear cell kidney cancer and studying the associations of SNPs and outcome.

Interestingly we have identified two SNPs in the VEGFR3 gene associated with worse response to sunitinib and one in the enzyme CYP3A5 associated with higher risk of dose reductions.
Additionally a second study in the same population showed that VEGFR3 SNPs were associated with a downexpression of the gene assessed by immunohistochemistry.

Finally we confirmed the findings from an external group associating an SNP in the IL8 gene with worse outcome.

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ABREVIATURAS

ADN: Ácido desoxirribonucleico

AMP: Adenosina monofosfato

CRCC: Carcinoma renal de células claras (CRCC)

VEGF: Vascular Endothelial Growth Factor

VEGFR: Vascular Endothelial Growth Factor Receptor

mTOR: mammalian Target Of Rapamycin

IL-2: Interleukina-2

EGFR: Epidermal Growth Factor Receptor

CAIX: Carbonic Anhidrase IX

HIF1 α: Hipoxia Inducible Factor

CTLA4: Cytotoxic-T-Lymphocites Antigen 4 associated

PD1: Programmed cell Death 1

PDL1: Programmed cell Death 1 Ligand

PI3K: Phospho Inositol 3 kinase

VHL: von Hippel Lindau

SG: Supervivencia Global

ILP: Intervalo Libre de Progresión

SLP: Supervivencia Libre de Progresión

SNP: Single Nucleotide Polymorphism

FLT1: fms-related tyrosine kinase 1

FLT3: fms-related tyrosine kinase 3

SOGUG: Spanish Oncology Genitourinary Group

NGS: Next Generation Sequencing (NGS)

GWAS: Genome Wide Association (GWAS)

UE: Unión Europea

RECIST: Response Evaluation Criteria In Solid Tumors

INTRODUCCIÓN

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1. El cáncer renal de células claras y su manejo clínico

El carcinoma renal representa la 6ª neoplasia en frecuencia, con una incidencia de 12 por 100.000 habitantes al año en España.[1]Aunque este término engloba distintos tipos histológicos, cada uno con un comportamiento completamente distinto, el 80% corresponde a los denominados tumores de células claras. El presente proyecto y los antecedentes aquí descritos se centran en este subtipo.

El carcinoma renal de células claras (CRCC) puede alcanzar la curación, mediante la resección quirúrgica, hasta en el 80% de los casos en estadios precoces. Sin embargo, una vez se desarrollan metástasis, se convierte en una enfermedad letal.

Actualmente existen 8 fármacos aprobados por las agencias reguladoras para el tratamiento del cáncer renal metastásico de células claras. Cuatro de estos agentes son inhibidores del receptor del factor de crecimiento del endotelio vascular (VEGFR del inglés Vascular Endothelial Growth Factor Receptor), uno es un anticuerpo monoclonal contra el factor de crecimiento del endotelio vascular (VEGF del inglés Vascular Endothelial Growth Factor), dos son inhibidores de los complejos enzimáticos formados por la proteína denominada “mammalian Target Of Rapamycin” (mTOR) y uno es una forma recombinante de una citoquina endógena (la interleukina-2 o IL-2).

Cada uno de estos agentes ha demostrado ser beneficioso para un subgrupo de pacientes, habiéndose obtenido un claro avance respecto a la situación de esta neoplasia hace 10 años.

Sin embargo, aún no somos capaces de identificar adecuadamente dichas poblaciones por lo que no podemos personalizar su tratamiento. Para ello, el descubrimiento de biomarcadores con una potencial aplicación a la práctica clínica es un paso esencial, y el objetivo final de la presente tesis doctoral.

2. El reto de la identificación de biomarcadores en CRCC

Tal y como describieron Danila et al hace unos años, los contextos en que un biomarcador puede condicionar la toma de decisiones médicas son:

- Detección, donde empleamos los marcadores como herramienta diagnostica

- Pronóstico, cuantificando las probabilidades de un evento (recurrencia, progresión o supervivencia)

- Predicción, identificando la probabilidad de respuesta a una terapia concreta

- Indicador de respuesta, como por ejemplo el PSA (lo que no necesariamente debe ir asociado a un beneficio clínico)

- Indicador de eficacia, como marcadores subrogados de una mayor supervivencia y extrapolando por tanto un beneficio clínico.

- Marcadores de Resistencia a un tratamiento, aquellos parámetros biológicos que determinan el desarrollo de un fallo del tratamiento (por ejemplo las mutaciones secundarias de la proteína denominada Epidermal Growth Factor Receptor (EGFR)[2]

Cada una de estas clases de biomarcadores presentará un mecanismo de acción y una relación con la enfermedad diferentes. Si bien una búsqueda sistemática debería producir resultados satisfactorios en las 6 categorías (detección, pronóstico, predicción, indicador de respuesta, indicador de eficacia o resistencia) la realidad es que en cáncer renal no disponemos de ninguno de ellos.

Las razones para este fracaso son diversas e incluyen la ausencia de candidatos bien definidos, los pequeños tamaños muestrales que suelen presentar los estudios de biomarcadores, el corto seguimiento y la ausencia de series de validación independiente. Además, las barreras legales para la recogida sistemática de muestras biológicas y la heterogeneidad tumoral, son otros obstáculos relevantes en esta enfermedad. [3]

2.1 La problemática de la recogida de muestras y datos clínicos

Dos "colecciones" diferentes deben recogerse adecuadamente para permitir identificar un biomarcador en cáncer renal: los datos clínicos y las muestras biológicas.[4]

Algunos de los problemas encontrados en la realización de este trabajo, así como las soluciones aplicadas, se mencionan a continuación:

- Dificultades éticas y legales. Nuestra capacidad, cada vez mayor, de analizar y utilizar muestras biológicas en investigación, plantea la cuestión de dónde debe situarse el límite entre lo que técnicamente somos capaces de realizar y lo que éticamente deberíamos llegar a hacer.

La ausencia de referencias y límites claros en un campo en constante evolución, deja a los investigadores la responsabilidad de averiguar qué es factible, a los comités de ética qué es moralmente tolerable y a los pacientes qué es para ellos aceptable.

La forma en que actualmente se realiza el proceso del consentimiento informado remeda al que, durante décadas, se ha seguido para preservar la autonomía de los pacientes a la hora de aplicar una prueba diagnóstica o iniciar un tratamiento.

Así, el médico informa de cómo se obtendrán las muestras, cómo serán almacenadas, cómo se analizarán y las potenciales implicaciones de los resultados. Sin embargo, en ocasiones, aparecen nuevas tecnologías en el transcurso de un estudio que no estaban disponibles cuando éste se diseñó o bien los análisis preliminares apuntan a nuevas líneas de trabajo no recogidas en el consentimiento original. Estos cambios, pueden conllevar repercusiones no previstas tanto para el sujeto incluido en un estudio como para sus familiares, que pueden desconocer por completo la investigación en marcha.

Para evitar, o al menos reducir al máximo los potenciales conflictos éticos, deberían seguirse unas directrices generales a la hora de llevar a cabo cualquier trabajo trasnacional:

Primero, en el consentimiento informado debe plantearse la posibilidad de que surjan nuevos estudios y resultados no recogidos originariamente.

Segundo, debe preguntarse al paciente de forma específica sobre su deseo de conocer los potenciales resultados de la investigación.

Tercero, la anonimización de las muestras es una medida muy útil que permite conciliar la privacidad del individuo con una investigación dinámica capaz adaptarse a los nuevos conocimientos.

En nuestro caso, aplicamos las tres premisas anteriores a los trabajos que forman parte de esta tesis lo que nos ha permitido desarrollar una línea de trabajo completa y sólida, con múltiples estudios adicionales, algunos aún en desarrollo.

-Dificultades en la recogida de muestras biológicas. Existe una serie de aspectos técnicos relacionados con la adquisición de tejido, su procesamiento y análisis, que pueden afectar a los resultados de un estudio y, al menos parcialmente, explicar los resultados contradictorios entre trabajos similares. Por ejemplo, se ha establecido cómo variables peroperatorias como la duración del tiempo de anestesia o variaciones en la tensión arterial, pueden por sí solas afectar a algunos marcadores biológicos como el estado de fosforilación de vías intracelulares.[5]

Obviamente, estos parámetros no pueden ser modificados pero sí deben hacerse todos los esfuerzos para que la recogida de las muestras sea lo más sistemática posible, especialmente cuando materiales muy "sensibles", como el RNA, son el objetivo de un estudio concreto.

Así, las muestras no deberán permanecer a temperatura ambiente durante periodos largos y deberían ser incluidas en medios de preservación específicos con la máxima celeridad. Además, está bien establecido que los métodos de fijación son clave en la adecuada preservación de las muestras. Por ejemplo, se ha determinado que el tipo de fijación puede producir la aparición de nuevas mutaciones en el DNA y afectar la antigenicidad de un tejido.[6]

Por tanto, la estandarización de los procedimientos de recogida de muestras, siguiendo las recomendaciones internacionales, es fundamental y siempre deben tenerse en mente

este tipo de artefactos. Otra forma de mejorar la fiablidad de los resultados es la validación externa de los hallazgos en cohortes independientes, punto este exigido por todas las publicaciones de prestigio,.

En nuestro caso, la mayoría de los trabajos se realizaron en muestras de sangre periférica extraídas "ad hoc" para este proyecto. Además, como se verá en la discusión, hemos conseguido validar parte de nuestros hallazgos en series externas.

- Dificultades en la recogida de datos clínicos. La recogida de datos clínicos es un punto clave en la investigación traslacional y, en ocasiones, aún más complejo que la recogida de muestras biológicas.

Las historias clínicas no están siempre accesibles y la obtención de información a partir de dichos archivos es laboriosa. Así algunos aspectos considerados fundamentales para una investigación pueden estar mal recogidos o incluso faltar en los evolutivos del médico por carecer de transcendencia clínica. De ahí que siempre deba preferirse el diseño de trabajos prospectivos en los que no solo el investigador clínico podrá tener en mente los objetivos del estudio y recoger adecuadamente los parámetros requeridos, sino que cualquier duda podrá resolverse con relativa facilidad mediante la consulta al investigador o al propio paciente.

Otra medida útil para conseguir una información clínica de calidad, es la monitorización externa de las historias. Aunque muchas veces no es posible debido a su elevado coste, representa una de las mejores formas de asegurarnos que evitamos sesgos en la forma en que los archivos clínicos son revisados e interpretados.

- Heterogeneidad tumoral. Por cuestiones obvias, todos los estudios de biomarcadores se realizan, casi por definición, sobre volúmenes tumorales pequeños. Así, la toma de muestras múltiples es generalmente inviable sencillamente porque choca con la necesidad de preservar el bienestar y seguridad del paciente. Por tanto, debemos asumir que la muestra con la que trabajamos es representativa de todo el tumor y esto no siempre es así, especialmente en cáncer renal.

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Ya los primeros exámenes al microscopio óptico, con el empleo de la inmunohistoquímica, revelaron que el aspecto morfológico de estos tumores y la distribución de las tinciones, variaba de una zona a otra.

En la misma línea, en 1985 un estudio de citometría de flujo en el que se determinó la ploidía del ADN de 25 casos de CRCC, se encontró una marcada heterogeneidad en uno de los casos.[7]

Igualmente, en 1993 un estudio con el anticuerpo radiomarcado G250 sobre la distribución de dicha proteína en cánceres renales, demostró una amplia variabilidad entre distintas áreas de los tumores de 16 pacientes con CRCC.[8]

Sin embargo, uno de los trabajos más destacados en este campo ha sido el comunicado por Gerlinger et al en 2012.[9] En él se demostró una marcada heterogeneidad genética entre distintas áreas de un mismo tumor primario y con respecto a sus metástasis. Obviamente, este trabajo reabre el dilema de hasta dónde deberíamos llegar en la realización de muestreos tumorales y biopsias múltiples para caracterizar adecuadamente cada caso desde el punto de vista molecular.

Sin embargo, examinando en detalle dicho estudio, los autores nos proponen una hipótesis elegante que denominan: "evolución convergente". Así, los cambios genéticos que las distintas lesiones en distintas localizaciones presentan, no se producen al azar, sino que guardan un cierto "orden". Es decir, aunque distintas regiones presentan mutaciones diferentes, muchas lo hacen en los mismos genes y con una repercusión funcional parecida. Esto apunta a que las alteraciones iniciales comunes a todas las localizaciones metastásicas, condicionarán de algún modo el patrón de la evolución posterior del tumor a nivel genético.

Además, no todas las alteraciones tienen el mismo peso en el curso de la enfermedad y muchas serán "pasajeras" o carecerán de interés de cara al manejo clínico.

En definitiva, uno de los mayores retos de la investigación traslacional de los próximos años será "ponderar" el valor y la relevancia de la multitud de hallazgos que las técnicas "ómicas" nos van a proporcionar.

Solo de esta manera podremos interpretar de una forma práctica la ingente cantidad de datos que vamos a ser capaces de obtener de cada caso.

3 Biomarcadores en cáncer renal: estado del arte

Aunque es bien conocido el impacto que los fármacos antiangiogénicos tuvieron en la evolución y tratamiento del cáncer renal de células claras, igualmente notorio fue su efecto sobre la producción científica en esta enfermedad.

Al convertirse en uno de los primeros tumores donde las terapias biológicas demostraron su superioridad frente a las aproximaciones clásicas, se desató un cierto entusiasmo en el desarrollo de estrategias para la personalización de los tratamientos.

Si bien nuestro grupo ha tenido la fortuna de participar en muchos de esos trabajos, explorando áreas tan dispares como la determinación de niveles de células endoteliales circulantes o los perfiles de miRNAs, en esta introducción nos ceñiremos a aquellas áreas relacionadas con los estudios que conforman la presente tesis doctoral. En concreto, repasaremos los resultados de trabajos en los que se estudiaron determinaciones inmunohistoquímicas en tejido tumoral, alteraciones genéticas tumorales (como las variaciones en el número de copias de genes) y los polimorfismos.

3.1 Biomarcadores basados en la expresión proteica determinada por inmunohistoquímica en pacientes tratados con inmunoterapia.

En 2003 Bui et al publicaron una serie retrospectiva de casos con CRCC metastásico en la que observaron una asociación entre los niveles de expresión del enzima Anhidrasa Carbónica IX (CAIX de sus siglas en inglés) y una mayor supervivencia.[10] En dicha serie se estableció como punto de corte una tinción superior al 85% para considerar una muestra como "positiva". La expresión de CAIX está directamente regulada por el Factor Inducible por Hipoxia1α (HIF1 α de sus siglas en inglés), lo que explicaría sus elevados niveles en cáncer renal.

Dos años más tarde, Atkins et al llevaron a cabo un estudio de casos y controles basado en datos clínicos y muestras patológicas de 66 pacientes con CRCC metastásico

procedentes de una serie de 231 casos incluidos en diferentes ensayos con IL-2 en una misma institución.[11] Utilizando el punto de corte previamente propuesto por Bui et al los autores encontraron que el porcentaje de pacientes que alcanzaban respuesta con esta terapia era mayor en los casos considerados como con sobreexpresión de CAIX (p=0.04).

Curiosamente, cuando combinaron la tinción para CAIX con un modelo predictivo diseñado previamente por el mismo grupo, el 96% de los respondedores fueron incluidos en la cohorte de buen pronóstico.[12]

En base a estos alentadores resultados, lo mismos investigadores desarrollaron el ensayo SELECT, que pretendía validar de forma prospectiva un score que, combinando la expresión por inmunohistoquímica de CAIX con una escala anatomopatológica y el denominado "University of California at Los Angeles Survival After Nephrectomy and Immunotherapy (SANI) score", permitiera seleccionar los pacientes que obtendrían mayor beneficio del tratamiento con IL-2 endovenoso. Desgraciadamente el modelo falló a la hora de predecir respuesta al tratamiento.[13]

Mucho más recientes son las comunicaciones en relación a las nuevas inmunoterapias, los denominados "checkpoint inhibitors" [CTLA4 (linfocitos T citotóxicos antígeno 4 asociado), PD1 (muerte celular programada 1) y PDL1 (muerte celular programada ligando 1)].[14,15,16] Sin embargo, y a pesar de los excelentes resultados clínicos obtenidos con compuestos como nivolumab, los primeros intentos de buscar un biomarcador de respuesta (fundamentalmente la tinción por inmunohistoqímica para las proteínas PD1 y PDL1) han resultado infructuosos.

3.2 Biomarcadores basados en la expresión, determinada por inmunohistoquímica, de CAIX, y PI3K y la presencia de mutaciones en el gen VHL en pacientes tratados con agentes antiangiogénicos.

Las mutaciones /delecciones/metilaciones del gen von Hippel Lindau (VHL) son las alteraciones moleculares más frecuente en cáncer renal, afectando en torno al 90% de los CRCC. [17] Además su papel central en este tumor está mi bien establecido y constituyó el racional para el desarrollo de las terapias antiangiogéncas en esta neoplasia.[18]

En 2006 Rini et al publicaron los resultados de un pequeño trabajo retrospectivo realizado en 43 casos estudiando una posible asociación entre la presencia de alteraciones en VHL y respuesta a tratamiento antiangiogénico.[19] Los resultados fueron prometedores, dando lugar a un segundo proyecto en el que se analizaron 123 pacientes.[20] Desgraciadamente los resultados finales no confirmaron las expectativas iniciales y solo en un subestudio, a posteriori, donde se seleccionó el tipo de mutaciones en VHL a analizar, alcanzó las significación estadística.

Otro trabajo de Peña et al investigó igualmente la asociación entre las alteraciones del gen VHL y los resultados clínicos obtenidos en pacientes con CRCC tratados con sorafenib dentro de su ensayo pivotal (TARGET). Este ensayo incluía pacientes en progresión a primera línea con citoquinas.[21]. Se consiguieron analizar 68 casos de los 903 originalmente incluidos en el ensayo, pero solo se realizó una secuenciación del gen, no determinaciones de su metilación. De los casos analizados, 35 portaban alguna mutación en VHL, pero sin relación con la respuesta al tratamiento.

En base a la posible capacidad de la anhidrasa carbónica IX para predecir respuesta a inmunoterapia, comentada en el apartado anterior, Choueiri et al realizaron un estudio unicéntrico en pacientes con CRCC tratados con sorafenib, valatinib o bevacizumab.[22] De una cohorte inicial de 118 pacientes, 94 fueron incluidos en el análisis y de estos 65 (69%) se consideraron como CAIX positivos (definidos como con una tinción por inmunohistoquímica >85% de las células tumorales). Aunque no se pudo establecer ninguna asociación entre la expresión de CAIX y respuesta al tratamiento, se observó una tendencia a una mejor evolución en los pacientes con niveles altos.

Dicha observación propició un último estudio en el que se analizó la expresión de CAIX en muestras del ensayo TARGET con sorafenib. En esta ocasión se obtuvo material viable de 133 de los 903 casos iniciales. Sin embargo, a pesar de explorar diferentes umbrales de sensibilidad, ninguna asociación pudo establecerse con la reducción del tamaño tumoral ni con la supervivencia.

Finalmente, el estado de activación de la vía de la PI3K y su valor en la predicción de respuesta a antiangiogénicos fue evaluada por Jonasch et al en pacientes tratados

también con sorafenib.[23] De una cohorte de 80 casos incluidos en un ensayo fase II, 40 contaban con tejido tumoral suficiente para su estudio. En un análisis multivariante, la fosforilación de AKTS473 fue el predictor más potente de una mayor supervivencia. El mismo autor, en un segundo estudio en pacientes a los que se administró bevacizumab de forma previa a la nefrectomía, analizó los niveles de activación de 37 proteínas en 42 pacientes.[24] Los niveles elevados de (AMP)kinasa se asociaron con una mayor supervivencia libre de progresión y una tendencia hacia una mayor supervivencia.

3.3 Variaciones en el número de copias de genes.

En la última década diversos investigadores han demostrado que existen variaciones groseras en el número de copias de algunos genes en cáncer renal y que estos cambios proporcionan una información pronóstica.[25,26,27,28]

Sin duda, el mejor ejemplo de este tipo de alteraciones lo representa la pérdida del brazo corto del cromosoma 3. Esta pérdida da lugar no solo a una delección de una copia de gen VHL sino, también, a una haploinsuficiencia de genes próximos como SETD2, PBRM1 y BAP1.[29,30,31,32]

Otras variaciones frecuentes en el número de copias de genes incluyen la ganancia de la región 5q, asociada a un mejor pronóstico, y las delecciones 14q y 9p y la ganancia 8q asociadas a una evolución desfavorable.

Así la haploinsuficiencia del gen HIF1A, característica de la pérdida de la región 14q, ha sido considerada de singular importancia por diferentes autores y la ganancia de copias del gen MYC, contenido en el cromosoma 8, también parece ser relevante. [25,33,28] Otra alteración ampliamente estudiada ha sido la pérdida del cromosoma 9p que podría tener importancia pronóstica, debido a la presencia de los genes CAIX y CDKN2A.

Aunque queda mucho por entender sobre las implicaciones de las variaciones en el número de copias de genes en cáncer renal, su relación con la respuesta a algunos tratamientos, como pazopanib, ha sido sugerida por algunos autores. [34]

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3.4 Polimorfismos

La capacidad que tienen los polimorfismos de condicionar tanto la eficacia como la toxicidad de algunos fármacos en un individuo es la principal característica por la que han despertado tanto interés en cáncer renal.[35,36] Dado que los fármacos antiangiogénicos tienen como principal diana las células endoteliales sanas del huésped y no el tumor en sí, parece lógico pensar que estas variantes genéticas pueden jugar un papel relevante en la resistencia y sensibilidad a dichos tratamientos.

Además, estos potenciales biomarcadores solo requieren una extracción de sangre periférica para su determinación, su presencia es homogénea en todas las células del organismo y no se modifican en el tiempo.

Todos estos puntos han favorecido ampliamente su estudio, siendo probablemente uno de los campos en que más publicaciones se han generado en cáncer renal en la última década.

Sin embargo, a pesar del interés despertado, existen dificultades metodológicas comunes a todos los estudios que podrían explicar por qué los resultados no han podido incorporarse a la clínica asistencial.

Así, existen notables diferencias entre las distintas publicaciones en cuanto a los polimorfismos analizados, las poblaciones incluidas y los fármacos recogidos. Todo ello ha impedido alcanzar conclusiones sólidas y consistentes.

A continuación revisaremos los datos más relevantes, agrupados por fármacos para facilitar su comprensión.

Bevacizumab. En el caso de los estudios sobre el papel de los polimorfismos en pacientes tratados con bevacizumab, los esfuerzos se han centrado en el factor soluble (VEGF-A) por ser éste la diana principal del anticuerpo.

Los primeros trabajos, realizados en cáncer de mama, encontraron que los SNPs rs1570360 (-1154G→A), rs699947 (-2578A→C) y rs3025039 (936C→T) se asociaban

a una mayor supervivencia global (SG), mientras que el denominado rs2010963 (-634G→C) lo hacía con toxicidad. [37,38]

Otros trabajos, esta vez en cáncer colorectal, encontraron una asociación entre el cambio rs1570360 (-1154G→A) y eficacia, e identificaron otro SNP, rs833061 (-1498C→T), asociado con supervivencia. [39,40]

Por ultimo, en un estudio realizado en cáncer de ovario con ciclofosfamida más bevacizumab, el SNP VEGF-A rs3025039 (936C→T) demostró una tendencia a asociarse con un mayor Intervalo Libre de Progresión (ILP) (P= 0.0.061).[41]

Dada su utilización minoritaria en cáncer renal, son relativamente escasos los trabajos sobre bevacizumab en esta patología. Quizá el más interesante fue el comunicado por Lambrechts et al donde una cohorte de pacientes del estudio AVOREN (ensayo clínico fase III que demostró la utilidad del fármaco en CRCC) se empleó para validar los hallazgos de otra cohorte, procedente del ensayo AVITA (bevacizumab en cáncer de páncreas). Este estudio encontró que un SNP en el gen VEGFR1 (FLT1), denominado rs9582036, se asoció a un mayor ILP (HR 1·81, 1·08-3·05; p=0·033).

Sorafenib. La mayoría de estudios sobre sorafenib y polimorfismos se han centrado en la toxicidad del fármaco más que en su eficacia. Así, un primer trabajo que analizó de forma retrospectiva una cohorte de 120 casos tratados con sorafenib junto a una segunda cohorte de 41 casos tratados con el mismo fármaco en los que se determinaron los niveles de bilirrubina antes y después de iniciada la terapia, señaló la posibilidad de que algunas variantes en los genes UGT1A1, UGT1A9 y ABCC2 (implicados en la aparición de hiperbilirrubinemia tóxica, la metabolización del fármaco y su transporte) podrían ocasionar una mayor concentración bajo la curva de sorafenib y por tanto una mayor exposición al mismo.[42]

En otro estudio, que analizó 178 casos con diferentes tumores que recibieron sorafenib o bevacizumab, se determinó que la presencia del SNP en VEGFR2 (rs1870377) se asociaba a una mayor incidencia de síndrome mano-pié e hiperbilirrubinemia.[43]

Sunitinib. La amplia utilización de este fármaco como uno de los estándares en primera línea en CRCC metastásico, lo ha convertido en el centro de la mayoría de los estudios sobre SNPs.

En lo concerniente a toxicidad vanErp et al estudiaron 31 polimorfismos en 219 casos diagnosticados de cáncer renal o tumors del estroma intestinal (GIST) tratados con sunitinib.[44] La asociación más significativa en el análisis multivariante se dio entre la aparición de leucopenia y el SNP en FLT3 (VEGFR3) rs1933437(T227M) y CYP1A1 rs1048943 (I462V),mucositis y CYP1A1 rs1048943 y cualquier toxicidad > 2 y VEGFR2 rs2305948 (V297I).

En cuanto a la hipertensión, Kim et al estudiaron 63 pacientes con CRCC metastásico y encontraron una asociación con el SNP rs2010963 (-634G→C) situado en el promotor del gen VEGF.[45]

Finalmente, en un intento por poder determinar alguna asociación con eficacia, van der Veldt et al estudiaron la relación entre la SLP y SG con diferentes SNPs en 136 casos de CRCC, entre los que se incluyeron los pacientes estudiados previamente por van Erp.[46]

Aunque encontraron asociaciones entre supervivencia y los SNPs CYP3A5 rs776746 y VEGFR2 rs1870377 y dos haplotipos de los genes NR1I3 y ABCB1 los resultados no fueron controlados para multiple testing.

Pazopanib. Xu et al comunicaron en 2011 el primer trabajo sobre polimorfismos y pazopanib.[47] Estudiaron 27 SNPs en 397 casos de CRCC incluidos en tres ensayos clínicos con este fármaco. Dos polimorfismos en el gen IL8, rs1126647(2767A→T) y rs4073(-251T→A) y en HIF1A, rs11549467(A588T), demostraron una asociación significativa con un corto ILP (P< 0.009, P<0.01 y P<0.03, respectivamente). Además, identificaron cinco polimorfismos en los genes HIF1A, NR1 │2 yVEGFA asociados significativamente con respuesta (P<0.05).

Axitinib El ensayo pivotal que comparó este fármaco con sorafenib en segunda línea de CRCC (el llamado "ensayo AXIS"), llevaba asociado un estudio traslacional sobre SNPs. En dicho estudio se encontraron tres SNPs en el gen VEGF-A, rs699947,

rs833061 y rs1570360, estadísticamente asociados con ILP. Esta asociación fue más clara en el brazo de los pacientes tratados con axitinib.[48]

Curiosamente, un estudio sobre la farmacocinética del compuesto, realizado en 315 voluntarios, determinó que la presencia de 15 SNPs potencialmente relacionados con el metabolismo del fármaco, no se asoció con una mayor exposición al tratamiento.[49]

En definitva, la búsqueda de biomarcadores en cáncer renal capaces de predecir tanto toxicidad como beneficio tras la exposición a fármacos antiangiogénicos, ha sido un área de intenso trabajo por parte de múltiples grupos internacionales en los últimos años.

Nuestra principal línea de investigación, el estudio del efecto de los polimorfismos, se desarrolló en paralelo con muchos de los trabajos aquí comentados. A pesar de establecerse un ambiente competitivo, esto no nos impidió participar en múltiples iniciativas cooperativas tanto de ámbito europeo como transcontinental.

De esta manera, no solo hemos podido protagonizar alguna de las publicaciones más relevantes en este campo sino que, además, hemos sentado las bases para liderar proyectos de gran envergadura en la era de la inmunoterapia en cáncer renal.

OBJETIVOS

OBJETIVOS

El objetivo principal de esta Tesis fue la identificación de marcadores predictivos de respuesta al tratamiento antiangiogénico en pacientes con cáncer renal de células claras. Para ello nos centramos en estudiar las variaciones genéticas del ADN germinal de los pacientes y su asociación con la eficacia y toxicidad del tratamiento. Asimismo, caracterizamos en el tejido tumoral la expresión proteica de diversos genes clave en el proceso de hipoxia y analizamos su asociación con la respuesta y supervivencia de los pacientes tratados con estos fármacos. El potencial de estos estudios, y el objetivo final de esta Tesis, es generar conocimiento que pueda ayudar a la mejora y personalización del manejo clínico de los pacientes con CRCC.

Este objetivo general puede desglosarse en los siguientes objetivos específicos:

- Objetvio 1: Establecer si existen diferencias significativas en la respuesta y supervivencia libre de progresión entre pacientes con cáncer renal de células claras metastásicos, tratados en primera línea con un fármaco antiangogénico (sunitinib), portadores de distintos polimorfismos en genes clave para la acción de este fármaco.
- Objetivo 2: Estudiar la asociaciación de dichos polimorfismos con toxicidades clínicamente relevantes en dichos pacientes.
- Objetivo 3: Caracterizar los niveles de expresión proteica de genes relacionados con la hipoxia en el tejido tumoral y estudiar su asociación con la evolución clínica de estos pacientes.

ARTÍCULOS

ARTÍCULO 1: Single nucleotide polymorphism associations with response and toxic effects in patients with advanced renal-cell carcinoma treated with first-line sunitinib: a multicentre, observational, prospective study.

Autores: Garcia-Donas J, Esteban E, Leandro-García LJ, Castellano DE, del Alba AG, Climent MA, Arranz JA, Gallardo E, Puente J, Bellmunt J, Mellado B, Martínez E, Moreno F, Font A, Robledo M, Rodríguez-Antona C.

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Resumen: Sunitinib es un inhibidor de tirosin kinasa con una demostrada eficacia en cáncer renal. Sin embargo, algunos pacientes no responden a este fármaco o desarrollan efectos secundarios importantes que obligan a reducir la dosis. Dado que no existen biomarcadores capaces de predecir sensibilidad / resistencia o toxicidad al fármaco, desarrollamos un estudio observacional prospectivo en el que incluimos pacientes en edad adulta afectados de carcinoma renal de células claras en 15 instituciones españolas miembros del grupo SOGUG (Spanish Oncology Genitourinary Group).

Los pacientes fueron tratados con sunitinib de acuerdo a la práctica asistencial de cada centro y siguiendo las pautas de revisiones locales.

Se estudiaron la tasa de respuesta, según criterios RECIST 1.0, la supervivencia libre de progresión y supervivencia global y su asociación con 16 polimorfismos en 9 genes VEGFR2 (rs2305948 y rs1870377), VEGFR3 (rs307826, rs448012 y rs307821), PDGFR-α (rs35597368), VEGF-A (rs2010963, rs699947, y rs1570360), IL8 (rs1126647), CYP3A4 (rs2740574), CYP3A5 (rs776746), ABCB1 (rs1045642, rs1128503 y rs2032582) y ABCB2 (rs2231142).

El estudio de las asociaciones se realizó mediante análisis uni y multivariante (incluyendo factores pronósticos clínicos como covariables). Ajustamos los resultados para multiplicidad (multiple testing) usando la corrección de Bonferroni (el método más

exigente). Se consideraron como significativos valores inferiores a una p menor de 0.031.

Resultados: Entre el 10 de octubre de 2007 y el 13 de diciembre de 2010 se incluyeron 101 pacientes. 95 de ellos fueron incluidos en el análisis de toxicidad y 89 en el de eficacia. Dos de los polimorfismos en el gen VEGFR3 se asociaron a una menor SLP en el análisis multivariante rs307826 (hazard ratio [HR] por alelo 3·57, 1·75-7·30; p(no-ajustado)=0·00049, p(adjustado)=0·0079) y rs307821 (3·31, 1·64-6·68; p(no-adjustado)=0·00085, p(adjustado)=0·014). Además el polimorfismo en el gen CYP3A5*1 (rs776746) relacionado con un aumento de la actividad metabolizadora del enzima, se asoció en el análisis multivariante con un riesgo aumentado de reducción de dosis debido a toxicidad (HR por alelo 3·75, 1·67-8·41; p(no-adjustado)=0·0014, p(ajustado)=0·022). Ningún otro SNP se asoció a respuesta o toxicidad por sunitinib.

Interpretación: la presencia de determinados polimorfismos en los genes VEGFR3 y CYP3A5*1 pueden definir una subpoblación de pacientes con cáncer renal, con menor respuesta y tolerabilidad a sunitinib. En caso de confirmarse en series independientes, podrían plantearse estudios intervencionistas con otras terapias para estos casos.

Aportación personal: Jesús García-Donas fue el promotor de la hipótesis del estudio, siendo responsable de la redacción del protocolo y diseño del trabajo en colaboración directa con la Dra. Rodríguez Antona. Así mismo obtuvo los fondos para su realización y construyó, a través del grupo SOGUG, el consorcio de hospitales que finalmente reclutaron los casos.
También supervisó de forma directa la recogida de datos clínicos, participó en el análisis de los resultados y el proceso de escritura y presentación del artículo final para publicación.

Single nucleotide polymorphism associations with response and toxic effects in patients with advanced renal-cell carcinoma treated with first-line sunitinib: a multicentre, observational, prospective study

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Summary

Background Sunitinib is a tyrosine kinase inhibitor with proven efficacy in renal-cell carcinoma, but some patients do not respond or need dose reductions due to toxicity. Because there are no validated molecular predictors of response or toxicity to sunitinib, we aimed to identify genetic markers predictive of outcome and toxic effects.

Methods In our observational, prospective study we enrolled previously untreated adults (≥18 years) with clear-cell renal-cell carcinoma at 15 institutions in the Spanish Oncology Genitourinary Group in Spain. Patients received sunitinib according to local practice guidelines. We assessed RECIST response, progression-free survival (PFS), overall survival, and toxicity of sunitinib with 16 key polymorphisms in nine genes: *VEGFR2* (rs2305948 and rs1870377), *VEGFR3* (rs307826, rs448012, and rs307821), *PDGFR*-α (rs35597368), *VEGF-A* (rs2010963, rs699947, and rs1570360), *IL8* (rs1126647), *CYP3A4* (rs2740574), *CYP3A5* (rs776746), *ABCB1* (rs1045642, rs1128503, and rs2032582), and *ABCB2* (rs2231142). We assessed associations with efficacy and toxicity by use of univariable and multivariable analyses (with clinical factors associated with outcomes as covariates). We adjusted for multiplicity using the Bonferroni method; p values of less than 0·0031 before adjustment were deemed to still be significant after adjustment.

Findings We enrolled 101 patients between Oct 10, 2007, and Dec 13, 2010. 95 of these patients were included in toxicity analyses and 89 in the efficacy analyses. Two *VEGFR3* missense polymorphisms were associated with reduced PFS with sunitinib on multivariable analysis: rs307826 (hazard ratio [HR] per allele 3·57, 1·75–7·30; $p_{unadjusted}$=0·00049, $p_{adjusted}$=0·0079) and rs307821 (3·31, 1·64–6·68; $p_{unadjusted}$=0·00085, $p_{adjusted}$=0·014). The *CYP3A5*1* (rs776746) high metabolising allele was associated in a multivariable analysis with an increased risk of dose reductions due to toxicity (HR per allele 3·75, 1·67–8·41; $p_{unadjusted}$=0·0014, $p_{adjusted}$=0·022). No other SNPs were associated with sunitinib response or toxicity.

Interpretation Polymorphisms in *VEGFR3* and *CYP3A5*1* might be able to define a subset of patients with renal-cell carcinoma with decreased sunitinib response and tolerability. If confirmed, these results should promote interventional studies testing alternative therapeutic approaches for patients with such variants.

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Introduction

Sunitinib malate is an orally administered tyrosine kinase receptor inhibitor that targets VEGF receptors, platelet-derived growth factor receptors, KIT, FLT-3, colony stimulating factor-1 receptor, and RET. Compared with first-line interferon α, sunitinib improved progression-free survival (PFS; 5 months *vs* 11 months, p<0·001) in a randomised phase 3 study[1] of patients with advanced clear-cell renal-cell carcinoma, and sunitinib is presently a standard treatment option. These findings were supported by data suggesting a possible effect on overall survival (21·8 months with interferon α *vs* 26·4 months with sunitinib; p=0·051),[2] and an expanded access trial of more than 4500 patients worldwide had equivalent outcomes.[3] However, some important caveats remain. Absence of efficacy is noted in 20% of patients who develop early progression of the disease and adverse events that, although manageable in most cases, lead to dose suspensions in 8% of patients, reductions in 32%, and delays in 38%.[1] Because drug exposure has been correlated with efficacy, toxic effects might not only affect patients' quality of life but also lead to dose modifications that could jeopardise treatment outcomes.[4] Biomarkers that can identify patients at risk of resistance to sunitinib or toxic effects could lead to new therapeutic approaches and improved results. Although different mechanisms of resistance have been proposed[5] the genetic background of the patient could have an important role—especially for drugs such as sunitinib that interact with the microenvironment of the tumour and non-malignant

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endothelial cells instead of having a direct cytotoxic activity. Recent description of hypertension as a reliable marker of sunitinib outcome supports the hypothesis that sunitinib sensitivity and resistance could be, at least in part, a host-dependent condition.[6]

We aimed to identify single nucleotide polymorphisms (SNPs) in the pharmacokinetic and pharmacodynamic pathways of sunitinib that are associated with response and toxicity in patients with advanced clear-cell renal-cell carcinoma treated with first-line sunitinib.

Methods

Study design and patients

We undertook our observational prospective study at 15 hospitals participating in the Spanish Oncology GenitoUrinary Group (SOGUG). We enrolled consecutively attending adults (≥18 years) who had a pathologically confirmed diagnosis of renal-cell carcinoma with a component of clear-cell histology and local or distant advanced disease. Eligible patients had not received any systemic treatment for kidney tumour, including cytokines, and were scheduled for sunitinib in a daily clinical practice setting. The protocol was approved by the medical ethics review board of all participating institutions, and signed consent was obtained for all patients.

	Patients (N=95)
Median age at time of sunitinib treatment (years)	65 (42–87, 56–73)
Sex (male)	65 (68%)
ECOG score	
0	25 (26%)
1	56 (59%)
2	8 (8%)
3	0
Missing	6 (6%)
Previous nephrectomy	76 (80%)
Number of metastatic sites	
0	2 (2%)
1	27 (28%)
2	44 (46%)
3	16 (17%)
4	5 (5%)
6	1 (1%)
Common metastasis sites	
Lung	66 (69%)
Lymph nodes	43 (45%)
Bone	24 (25%)
Kidney	17 (18%)
Liver	13 (14%)
Risk factors*	
0 (favourable)	42 (44%)
1–2 (intermediate)	50 (53%)
≥3 (poor)	3 (3%)
Initial sunitinib dose	
50 mg	84 (88%)
37·5 mg	9 (9%)
25 mg	2 (2%)

Data are median (range, IQR) or n (%). *Risk groups according to Memorial Sloan-Kettering Cancer Center prognostic factors: ECOG performance status >1, high lactate dehydrogenase concentrations (>1·5-times the upper limit of normal), low serum haemoglobin concentration, high corrected serum calcium concentration (>2·5 mmol/L), and no nephrectomy.[17] ECOG=Eastern Cooperative Oncology Group.

Table 1: **Clinical characteristics of patients**

Procedures

Drug schedule, dose-reduction policy, and timing of radiological assessments were decided by the attending doctors in accordance with current local practice guidelines. Demographic and clinical data were recorded on specific case record forms and periodically reviewed by an external monitor. Samples were anonymised two times: first by attending doctors and then centrally (Spanish National Cancer Research Centre). Investigators did the molecular analysis masked to clinical data.

We assessed 16 SNPs in nine genes potentially relevant for sunitinib action, metabolism, and transport. We chose these polymorphisms on the basis of functionality evidence from previously reported associations, those leading to aminoacid changes, and with reported minor allele frequencies of more than 5%. The chosen polymorphisms potentially affecting sunitinib pharmacodynamics were located in genes encoding sunitinib targets and ligand: *VEGFR2* (rs2305948 and rs1870377), *VEGFR3* (rs307826, rs448012, and rs307821), *PDGFR-α* (rs35597368), and *VEGF-A* (rs2010963, rs699947, and rs1570360),[7] and in *IL8* (rs1126647).[8] Six of these SNPs were putatively functional missense polymorphisms in the receptors, three corresponded to SNPs previously associated with bevacizumab response and tumour prognosis (the *VEGF-A* 5′-untranslated region and promoter variants),[9,10] and one corresponded to a SNP previously associated with PFS in patients with renal cancer treated with pazopanib (the *IL8* 3′-untranslated region variant).[8] The polymorphisms potentially affecting sunitinib pharmacokinetics were located in genes relevant for its metabolism (*CYP3A4* [rs2740574] and *CYP3A5* [rs776746])[11] and transport (*ABCB1* [rs1045642, rs1128503, and rs2032582] and *ABCG2* [rs2231142]).[12] *CYP3A5*1* is a high-activity allele,[13] whereas the SNP in *CYP3A4*, the major sunitinib metabolizing enzyme, is a promoter variant with contradictory results published about its activity.[14] Concerning the transporters, the *ABCG2* variant has been reported to increase sunitinib exposure[15] and the three *ABCB1* SNPs have been repeatedly associated with an altered P-glycoprotein activity.[16]

We isolated DNA from peripheral blood with the FlexiGene DNA kit (Qiagen, Valencia, CA, USA) or saliva with the Oragene DNA self-collection kits (DNA Genotek Ottawa, ON, Canada), according to the manufacturers'

recommended protocols. We quantified final DNA concentration with PicoGreen (Invitrogen, Carlsbad, CA, USA) and genotyped SNPs with the KASPar SNP genotyping system (Kbiosciences, Hoddesdon, UK). We used the sequence Detection System 7900HT (Applied Biosystems, Foster City, CA, USA) for fluorescence detection and allele assignment.

Statistical analysis

We defined PFS as the time between the first day of treatment with sunitinib and the date of radiological progressive disease (PD), clear clinical evidence of PD, or death. Patients who had not progressed at database closure were censored at final follow-up. If the date of PD was unknown, we censored PFS at the last tumour assessment. Overall survival was defined as the time between the first day of sunitinib treatment and the date of death or last date of follow-up. Objective response was assessed by treating doctors, according to Response Evaluation Criteria in Solid Tumors (RECIST) criteria version 1.0, and classified as complete response, partial response, stable disease, or PD. Timing for assessments was dictated by individual institution policy. All adverse effects were graded by the attending doctors according to Common Terminology Criteria for Adverse Events version 3·0 (see webappendix p 1). We selected mucositis, hand-foot syndrome, hypertension, anaemia, and thrombocytopenia for analysis on the basis of clinical relevance and grading objectiveness, together with grade 3–4 adverse events. We also recorded adverse toxic events leading to dose reductions and the date on which they occurred.

We tested SNP genotypes against PFS and overall survival with Cox-regression analysis and against RECIST response with logistic regression analysis. We did a multivariable analysis by including clinical factors associated with PFS, overall survival, or response as covariates (clinical factors that were associated with $p<0·1$ with a specific variable were used as covariates for that specific variable). We allocated patients to favourable, intermediate, and poor prognosis groups according to the Memorial Sloan-Kettering Cancer Center (MSKCC) prognostic classification, and included this variable in the multivariable analysis. We did not include centre as a covariate because the number of patients recruited at all 15 institutions was small (≤17). We further analysed SNPs associated with PFS and overall survival in the multivariable analysis by the Kaplan-Meier method.

We assessed genotypes associated with an increased risk of sunitinib dose reduction caused by toxicity with Cox-regression modelling of the number of days of sunitinib treatment until the reduction of dose. Patients with no dose reductions were censored at final follow-up. For multivariable analysis, we used clinical factors associated with $p<0·1$ with dose reductions as covariates, and further analysed the SNPs associated to sunitinib dose reduction in the multivariable analysis with the Kaplan-Meier method. We did this analysis for the subset of patients that started treatment with 50 mg sunitinib and for all patients. We studied associations between specific sunitinib toxicities and genotypes with logistic regression analysis, with toxicity development as a dichotomous endpoint. The multivariable logistic regression analyses included clinical factors associated with the corresponding outcome as covariates (factors with $p<0·1$). We tested all genotypes with an additive genetic model.

See Online for webappendix

	Single nucleotide polymorphism	Variation	Patients*	Homozygous wild-type	Heterozygous	Homozygous variant	Minor allele frequency
VEGFR2	rs2305948 C>T	V297I	95	78	15	2	0·100
VEGFR2	rs1870377 T>A	Q472H	95	53	34	8	0·263
VEGFR3	rs307826 A>G	T494A	95	80	15	0	0·079
VEGFR3	rs448012 C>G	H890Q	94	32	48	14	0·404
VEGFR3	rs307821 G>T	R1324L	95	78	16	1	0·095
PDGFR-α	rs35597368 T>C	S478P	95	77	16	2	0·105
VEGF-A	rs2010963 G>C	5′-UTR	95	47	37	11	0·311
VEGF-A	rs699947 A>C	Promoter	95	27	47	21	0·468
VEGF-A	rs1570360 G>A	Promoter	95	45	41	9	0·311
IL8	rs1126647 A>T	3′-UTR	94	35	48	11	0·372
CYP3A4	rs2740574 A>G	Promoter	94	89	5	0	0·027
CYP3A5	rs776746 G>A	Splicing	94	82	12	0	0·064
ABCB1	rs1045642 C>T	I1145I	94	27	51	16	0·441
ABCB1	rs1128503 C>T	G412G	95	36	45	14	0·384
ABCB1	rs2032582 G>T	A893S	92	38	39	15	0·375
ABCG2	rs2231142 C>A	Q141K	95	85	10	0	0·053

UTR=untranslated region. *Patients successfully genotyped.

Table 2: **Polymorphisms genotyped and allele frequency**

	Progressive disease as best response			Progression-free survival			Overall survival		
	Hazard ratio (95% CI)	$p_{unadjusted}$	$p_{adjusted}$*	Hazard ratio (95% CI)	$p_{unadjusted}$	$p_{adjusted}$*	Hazard ratio (95% CI)	$p_{unadjusted}$	$p_{adjusted}$*
VEGFR2 rs2305948	0·40 (0·05–3·26)	0·39	..	0·75 (0·38–1·48)	0·40	..	0·82 (0·32–2·12)	0·69	..
VEGFR2 rs1870377	1·33 (0·47–3·70)	0·59	..	1·09 (0·68–1·74)	0·71	..	1·74 (0·91–3·32)	0·092	..
VEGFR3 rs307826	8·79 (1·92–40·28)	0·0051	0·082	3·57 (1·75–7·30)	0·00049	0·0079	1·77 (0·65–4·84)	0·26	..
VEGFR3 rs448012	0·88 (0·34–2·29)	0·79	..	1·12 (0·68–1·85)	0·66	..	1·36 (0·71–2·59)	0·35	..
VEGFR3 rs307821	7·14 (1·46–35·00)	0·015	0·25	3·31 (1·64–6·68)	0·00085	0·014	1·24 (0·41–3·75)	0·71	..
PDGFR-α rs35597368	0·85 (0·21–3·39)	0·82	..	1·18 (0·63–2·21)	0·61	..	0·91 (0·39–2·08)	0·82	..
VEGF-A rs2010963	0·94 (0·39–2·27)	0·90	..	0·96 (0·62–1·49)	0·86	..	1·08 (0·59–1·96)	0·80	..
VEGF-A rs699947	1·41 (0·61–3·26)	0·43	..	1·01 (0·68–1·51)	0·96	..	0·72 (0·40–1·27)	0·25	..
VEGF-A rs1570360	1·31 (0·55–3·09)	0·54	..	1·13 (0·75–1·70)	0·56	..	0·79 (0·44–1·44)	0·44	..
IL8 rs1126647	1·27 (0·46–3·51)	0·64	..	1·20 (0·76–1·89)	0·43	..	1·44 (0·77–2·71)	0·25	..
CYP3A4 rs2740574	<0·001 (NA)	0·99	..	1·24 (0·36–4·26)	0·74	..	0·69 (0·15–3·09)	0·63	..
CYP3A5 rs776746	<0·001 (NA)	0·99	..	1·46 (0·64–3·31)	0·37	..	0·62 (0·18–2·12)	0·45	..
ABCB1 rs1045642	0·98 (0·37–2·58)	0·96	..	0·77 (0·51–1·19)	0·24	..	1·05 (0·59–1·86)	0·86	..
ABCB1 rs1128503	1·25 (0·46–3·38)	0·67	..	1·42 (0·95–2·12)	0·089	..	1·75 (0·99–3·12)	0·055	..
ABCB1 rs2032582	1·09 (0·43–2·79)	0·85	..	1·27 (0·86–1·88)	0·23	..	1·47 (0·85–2·54)	0·17	..
ABCG2 rs2231142	2·96 (0·96–9·15)	0·99	..	2·98 (0·85–10·47)	0·088	..	1·45 (0·42–4·98)	0·56	..

Multivariable analyses for response, progression-free survival, and overall survival include MSKCC risk groups and sex as covariates. NA=not assessable. *Adjusted for multiplicity using Bonferroni's method for those with a statistically significant unadjusted p value.

Table 3: **Multivariable analysis of polymorphisms associated with response, progression-free survival, and overall survival in patients with renal-cell carcinoma treated with sunitinib**

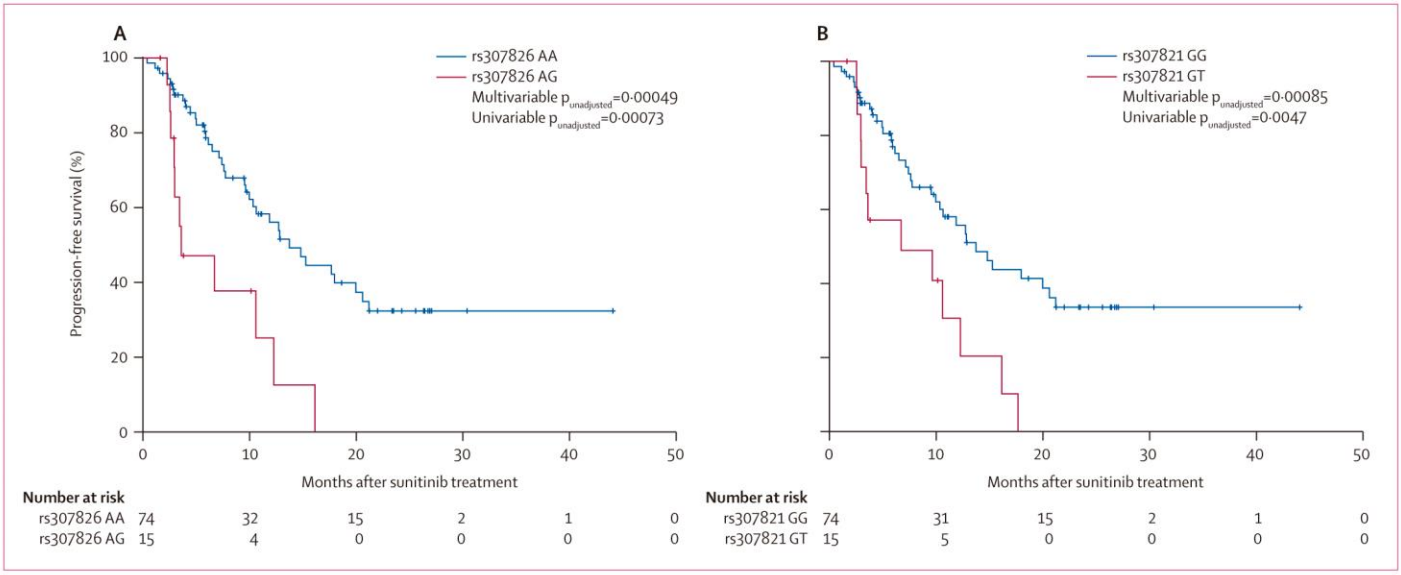

Figure 1: **Kaplan-Meier analysis of progression-free survival in patients with the rs307826 and rs307821 variants in *VEGFR3* with renal cancer after treatment with sunitinib**
(A) Patients grouped according to rs307826; median progression-free survival for wild type (AA) was 13·7 months (95% CI 9·7–17·7) and heterozygous (AG) was 3·6 months (0·0–8·7). (B) Patients grouped according to rs307821; median progression-free survival for wild type (GG) was 13·7 months (9·8–17·6) and heterozygous (GT) was 6·7 months (0·0–16·6). p values are from unadjusted multivariable analysis and from the unadjusted univariable log-rank test.

We retained missing data as missing apart from MSKCC prognostic factors. The most likely value, based on the data from the rest of the series, was assigned to these patients.

We corrected for multiple testing using the Bonferroni method. Because 16 polymorphisms were selected for the analysis, only unadjusted p values less than 0·0031 were therefore considered significant at the 95% confidence level.

All statistical analyses were done with RStudio version 0.93.

Role of the funding source

The sponsor of the study had no role in study design, data collection, data analysis, data interpretation, or writing of the report. JG-D, LJL-G, and CR-A had access to the raw data and JG-D, LJL-G, DEC, and CR-A had

final responsibility for the decision to submit for publication.

Results

We enrolled 101 patients between Oct 10, 2007, and Dec 13, 2010, and closed the follow-up database in May, 2011. We excluded six patients from the analyses (three did not provide blood or saliva samples, two had non-clear-cell kidney cancer, and one did not receive sunitinib). We confirmed malignant kidney tumours were present in six other patients, but subtype could not be established. We excluded these patients from the sunitinib efficacy analysis (ie, included 89 patients) but included them in the toxicity analysis (95 patients). Table 1 shows the clinical characteristics of enrolled patients; 66 (69%) of patients had multiple metastases. The minor allele frequencies of the 16 polymorphisms genotyped (table 2) were much the same as described elsewhere for whites (dbSNP database) and all SNPs were in Hardy-Weinberg equilibrium (p>0·05).

The variables we needed to assign an MSKCC score were missing for 18 patients (basal calcium concentration in 11 patients, performance status in six patients, and lactate dehydrogenase concentration in one patient). Some patients could be correctly assigned to an MSKCC group even with some missing data. After calculation of the most likely value for these patients, 15 were assigned to the good prognosis group and three were assigned to the intermediate prognosis group. Equivalent results were noted with and without the replacement of these missing factors, suggesting that the replacement did not skew the results. 92 (97%) of 95 patients were classified in the intermediate and favourable MSKCC risk groups (table 1). 94 patients were of white Spanish origin, one was of African (Moroccan) origin, and one was of Asian (Indian) origin.

After a median follow-up of 21·2 months (IQR 8·4–25·6, 95% CI 13·6–28·9), the median PFS of the 89 patients in the efficacy analysis was 12·3 months (IQR 5·8–>21·2, 95% CI 9·1–15·4) and 62 (70%) were alive by the time of the analysis. Objective response was assessed in 78 patients with measurable disease; one patient (1%) had a complete response, 36 (46%) had a partial response, 26 (33%) had stable disease, and 15 (19%) had PD.

In the toxicity analysis, the most common grade 1–3 events were asthenia in 67 (71%) of 95 patients, mucositis in 64 (67%), diarrhoea in 49 (52%), neutropenia in 40 (42%), and hand-foot syndrome in 39 (41%; webappendix p 1). 43 (45%) of 95 patients developed grade 3 adverse events and 47 (49%) required dose reductions because of toxic effects (webappendix p 1). No grade 4 toxic effects were reported.

Patients with high MSKCC risk factor scores and women had worse overall survival and a worse overall response than did those with low scores or men, but PFS did not differ between these groups (webappendix p 2). Women had a higher risk of needing a sunitinib dose reduction because of adverse events than did men (p=0·005; webappendix p 3).

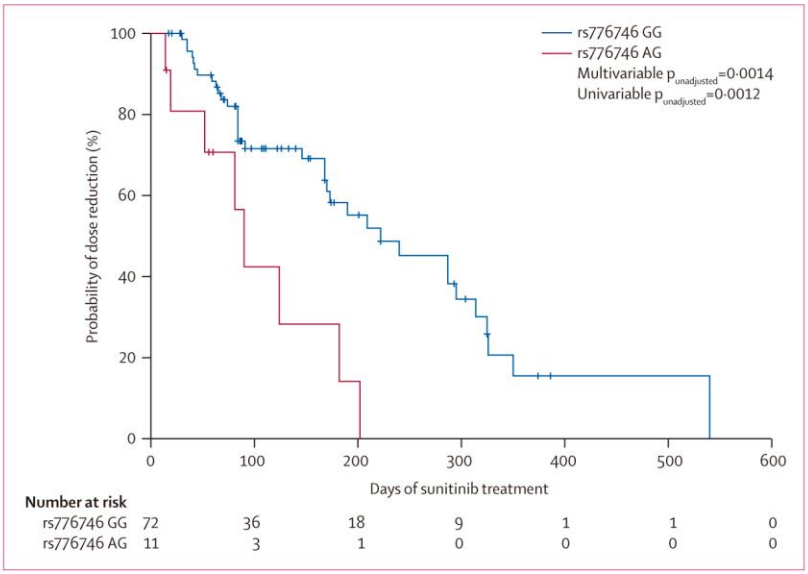

Figure 2: **Kaplan-Meier analysis of time on sunitinib treatment until dose reduction because of toxic effects for patients with the rs776746 variant in *CYP3A5***
84 patients included in the analysis had an initial sunitinib dose of 50 mg on a 4 weeks on, 2 weeks off schedule. One patient did not have genotype data for this single nucleotide polymorphism.

Table 3 shows results of the multivariable Cox regression analysis for the 16 polymorphisms genotyped. Two polymorphisms in *VEGFR3*, rs307826 and rs307821, were significantly associated with PFS (figure 1; table 3); these associations remained significant after Bonferroni correction for multiple testing. These *VEGFR3* polymorphisms are missense variants in moderate linkage disequilibrium (r^2=0·55) and few patients carried these variants independently. Thus, we constructed a model that shows the PFS of the patients grouped according to the genotype of both polymorphisms (webappendix p 5). Although not significant at the p<0·05 level, three additional polymorphisms in *ABCB1, ABCG2,* and *VEGFR2* (rs1128503, rs2231142, and rs1870377) showed a tendency to have worse response to sunitinib in terms of PFS or overall survival (table 3).

47 (94%) of the 50 observed dose reductions were attributable to adverse events (webappendix p 1). To detect factors relevant for dose reductions, we analysed the time of sunitinib treatment up to the reduction of dose because of toxicity in the 84 patients with 50 mg sunitinib starting dose. Women had dose reductions earlier than did men (p=0·005), and multivariable analysis showed *CYP3A5* rs776746 was an important risk factor (figure 2; table 4); this association remained significant after correction for multiple testing. We repeated this analysis for all 95 patients, and noted equivalent results (data not shown). Three variant alleles (*VEGFR2* rs1870377 and *VEGF-A* rs699947 and rs1570360) seemed significantly associated with an increased risk of hypertension (table 4), and two (*ABCB1* rs1128503 and rs2032582) seemed to confer protection. *VEGFR2* rs2305948 seemed associated with

For the **dbSNP database** see http://www.ncbi.nlm.nih.gov/projects/SNP/

	Toxicity-related dose reductions*			Hypertension			Hand-foot syndrome†			Mucositis		
	Hazard ratio (95% CI)	$p_{unadjusted}$	$p_{adjusted}$‡	Hazard ratio (95% CI)	$p_{unadjusted}$	$p_{adjusted}$‡	Hazard ratio (95% CI)	$p_{unadjusted}$	$p_{adjusted}$‡	Hazard ratio (95% CI)	$p_{unadjusted}$	$p_{adjusted}$‡
VEGFR2 rs2305948	1·12 (0·62–2·03)	0·71	..	1·09 (0·43–2·77)	0·85	..	3·94 (1·33–11·72)	0·014	0·22	1·81 (0·60–5·47)	0·29	..
VEGFR2 rs1870377	1·26 (0·80–1·98)	0·32	..	2·62 (1·32–5·20)	0·0058	0·092	1·55 (0·81–2·96)	0·19	..	1·04 (0·53–2·02)	0·91	..
VEGFR3 rs307826	0·95 (0·40–2·27)	0·90	..	1·31 (0·42–4·06)	0·64	..	1·27 (0·41–3·91)	0·68	..	0·68 (0·22–2·12)	0·51	..
VEGFR3 rs448012	0·85 (0·52–1·39)	0·51	..	1·13 (0·60–2·12)	0·72	..	0·81 (0·43–1·52)	0·51	..	1·25 (0·65–2·38)	0·50	..
VEGFR3 rs307821	1·31 (0·60–2·86)	0·49	..	2·01 (0·75–5·37)	0·17	..	0·84 (0·31–2·32)	0·74	..	0·58 (0·21–1·54)	0·27	..
PDGFR-α rs35597368	0·82 (0·38–1·74)	0·60	..	1·26 (0·51–3·10)	0·62	..	0·67 (0·25–1·82)	0·44	..	0·90 (0·36–2·27)	0·82	..
VEGF-A rs2010963	1·18 (0·76–1·85)	0·46	..	0·63 (0·32–1·20)	0·16	..	0·67 (0·36–1·26)	0·22	..	0·50 (0·27–0·95)	0·034	0·55
VEGF-A rs699947	0·97 (0·62–1·49)	0·87	..	2·43 (1·27–4·66)	0·0074	0·12	1·04 (0·58–1·87)	0·90	..	1·82 (0·96–3·44)	0·066	..
VEGF-A rs1570360	0·92 (0·57–1·51)	0·75	..	2·04 (1·05–3·96)	0·035	0·57	1·32 (0·69–2·51)	0·41	..	1·46 (0·74–2·90)	0·28	..
IL8 rs1126647	1·46 (0·92–2·32)	0·10	..	0·94 (0·49–1·80)	0·85	..	0·78 (0·41–1·49)	0·45	..	1·83 (0·91–3·69)	0·091	..
CYP3A4 rs2740574	2·27 (0·69–7·52)	0·18	..	1·31 (0·21–8·28)	0·77	..	2·06 (0·32–13·40)	0·45	..	>999 (NA)	0·99	..
CYP3A5 rs776746	3·75 (1·67–8·41)	0·0014	0·022	0·96 (0·27–3·48)	0·96	..	1·37 (0·40–4·76)	0·62	..	0·98 (0·27–3·55)	0·98	..
ABCB1 rs1045642	0·76 (0·45–1·27)	0·29	..	0·56 (0·29–1·09)	0·09	..	1·49 (0·78–2·83)	0·23	..	0·93 (0·49–1·78)	0·84	..
ABCB1 rs1128503	0·98 (0·62–1·56)	0·93	..	0·41 (0·20–0·81)	0·011	0·17	0·79 (0·43–1·46)	0·45	..	0·73 (0·39–1·35)	0·31	..
ABCB1 rs2032582	0·91 (0·57–1·46)	0·71	..	0·42 (0·21–0·84)	0·014	0·22	1·00 (0·55–1·82)	0·99	..	0·77 (0·42–1·41)	0·40	..
ABCG2 rs2231142	0·60 (0·14–2·52)	0·49	..	1·29 (0·34–4·93)	0·71	..	0·11 (0·01–0·92)	0·042	0·66	2·07 (0·41–10·39)	0·38	..

NA=not assessable. *Cox-regression analysis for time of sunitinib treatment until dose reduction in 84 patients with initial sunitinib doses of 50 mg; multivariable analysis includes sex as a covariate. †Multivariable analysis includes Memorial Sloan-Kettering Cancer Center (MSKCC) risk groups as a covariate. ‡Adjusted for multiplicity using Bonferroni's method for those with a statistically significant unadjusted p value.

Table 4: **Genetic factors associated with sunitinib dose reductions and toxic effects**

an increased risk of development of hand-foot syndrome and *ABCG2* rs2231142 seemed to confer protection. *VEGF-A* rs2010963 seemed associated with mucositis protection. However, these associations were not significant after adjustment for multiple testing (table 4).

Discussion

Our prospective assessment of SNPs as predictors of the efficacy and toxicity of first-line sunitinib in previously untreated patients with advanced clear-cell renal-cell carcinoma suggests that two *VEGFR3* missense polymorphisms are strongly associated with shorter PFS and one functional polymorphism in *CYP3A5* is associated with an increased risk of sunitinib dose reductions due to toxicity (panel). These associations, although not validated in an independent series, remained significant after correction for multiple testing. These data suggest that alternative treatment approaches for patients with these genetic variants should be promoted.

The increasing number of treatment options for kidney cancer—including four approved antiangiogenic drugs (sorafenib, sunitinib, bevacizumab, and pazopanib), at least three others in advanced stage of development (axitinib, tivozanib, and dovitinib), and various mTOR inhibitors—has made the identification of biological markers of response and toxicity for these drugs a key step in moving forward and improving patient care. Therefore, we aimed to identify polymorphisms as potential markers of sunitinib outcome, focusing on genetic variants that could alter the pharmacokinetics and pharmacodynamics of this drug (table 2). Our findings show that polymorphisms in *VEGFR3* and *CYP3A5* could account for part of the absence of response and low tolerability noted in some patients.

VEGFR3 is a transmembrane tyrosine kinase receptor of VEGF that has been mainly associated with lymphangiogenesis.[20] Although initially its expression was thought to be restricted to lymphatic vessels in adulthood, studies[21,22] have not only confirmed the expression of VEGFR3 in tumour blood vessels but also suggested VEGFR3 as a key mediator of other proangiogenic factors. Preclinical models have even reported that VEGFR3 could be more relevant than VEGFR2 for the development of lymphatic and distant metastases.[23] Furthermore, sunitinib administration can affect plasma-soluble VEGFR3 concentrations and this change has been associated with sunitinib efficacy in patients with kidney carcinoma.[24,25] Our study shows an association between two *VEGFR3* missense SNPs, rs307826 (T494A) and rs307821 (R1324L), with PFS (table 3). Conversely, van der Veldt and colleagues did not report a significant effect of rs307826 on PFS after sunitinib.[19] However, this discrepancy could be caused by differences in the patients studied, especially the use of previous medical treatments, which were not allowed in this study but were frequent (41% of the patients) in the van der Veldt study.[19] Because rs307826 and rs307821 are in moderate linkage disequilibrium (webappendix p 5), it is difficult to establish which is the causal SNP, or if both could have an effect on sunitinib response. rs307826 affects moderately conserved nucleotides and rs307821 affects weakly conserved ones, with T494A located in the

immunoglobulin homology domain 5 (D5) of VEGFR3, whereas R1324L is in the C-terminal region of the protein. Bioinformatic tools (such as SIFT and Align GVGD) in both cases predicted aminoacid changes that would affect protein function. However, because D5–D5 interactions might contribute to dimer stabilisation and activation of VEGFR3[26] and because our study shows a stronger association for this SNP with sunitinib response and PFS, we suggest that rs307826 (T494A) rather than rs307821 (R1324L) is the causal variant. Whether the *VEGFR3* variation could also be influencing prognosis is indeterminable, but its association with sunitinib overall response suggests a key role in drug efficacy.

The risk of dose reductions due to toxicity was significantly associated—even after correction for multiple testing—with *CYP3A5*1* (rs776746; figure 2 and table 4), which affects CYP3A5 enzyme expression.[13] CYP3A5 shares substrate specificity with CYP3A4,[27] which is the key enzyme catalysing sunitinib metabolism.[11] Thus, *CYP3A5*1* might metabolise sunitinib and result in an increased production of the active and longer-acting metabolite SU12662, leading to toxic effects. If confirmed, the large ethnic differences in *CYP3A5*1* allele frequency, which is more common in people of African or Asian origin than in people of European origin,[13] might be an underlying cause of the higher sunitinib toxicity reported in people from Asia.[28,29] Notably, van der Velt and colleagues reported an association between *CYP3A5*1* and improved PFS.[19] The sunitinib pharmacokinetic profile of carriers of *CYP3A5*1* should be investigated in future studies to clarify this point. For specific toxicities (table 4), the most relevant results corresponded to hypertension, with SNPs in *VEGFR2, VEGF-A,* and *ABCB1* having significant associations. Rini and colleagues[6] established hypertension as a sunitinib predictor of response and thus these SNPs could also be surrogate markers of sunitinib efficacy. In agreement with this, patients with the *ABCB1* rs1128503 variant T-allele, which is protective for hypertension, had worse overall survival and PFS than did patients without this allele (table 3). However, methodological differences in blood pressure assessment preclude a direct comparison between these two studies.

Only one study assessing sunitinib pharmacogenetics for toxicity[18] and one assessing efficacy have been published.[19] Although the investigators reported interesting results, both studies were regarded as exploratory and no correction for multiple testing was done. Other relevant differences from our study were that patients in these studies[18,19] were allowed to have previous treatments and that data were collected retrospectively and tumours other than renal cancer were included in toxic-effects analysis. Despite these differences, some of the associations reported had similar tendencies in our series (eg, *ABCB1* rs1128503 was linked with decreased PFS [p=0·089] and overall survival [p=0·055] and *VEGFR2* rs1870377 was linked with decreased overall survival [p=0·092]).

Our study had limitations. Schedule and dose modifications were not dictated by central protocol, and timing for radiological assessments was done according to each institution's policy. Thus, courses of treatment were not standardised for the study and outcomes were assessed with regard to present practice. Heng and colleagues[30] established haemoglobin, corrected calcium, performance status, time from diagnosis, neutrophils, and platelets as clinical predictors of outcome for metastatic renal-cell carcinoma treated with antiangiogenic drugs. However, our study started before this report was published and basal neutrophils were not recorded, precluding the application of this model to our population. The SNPs that we found to be associated with sunitinib outcome have a relatively low allele frequency (9% for rs307821, 8% for rs307826, and 6% for rs776746) decreasing the power of the study. Finally, our study did not include a prospective, external validation. Because our patients were mainly white, the relevance of these polymorphisms needs to be assessed in other ethnic groups. However, to ensure homogeneity of our data, we only included patients with clear-cell cancer component tumours, excluded previous treatment, and had data externally reviewed by an independent monitor. These factors are probably the major contributors to the robustness of our results, with statistically significant outcomes that persisted after adjustment for multiple testing.

Our results warrant pharmacokinetic studies to better understand the molecular mechanisms leading to dose reductions after sunitinib and further validation in independent series. If confirmed, these genetic variants could provide the basis for an individualised renal cancer treatment.

For **SIFT** see http://sift.bii.a-star.edu.sg/

For **Align GVGD** see http://agvgd.iarc.fr/agvgd_input.php

Panel: Research in context

Systematic review

We searched PubMed and American Society of Clinical Oncology databases for articles published up to August, 2007, without language restrictions with the search terms "renal carcinoma", "sunitinib", and "polymorphisms" and identified no studies about single nucelotide polymorphisms (SNPs) as markers of sunitinib outcome. We also searched PubMed to identify genes relevant for sunitinib pharmacokinetic and pharmacodynamic pathways. We chose variants in those genes of interest after revising known polymorphisms listed in the dbSNP database. During recruitment, one retrospective study about polymorphisms associated with sunitinib-induced toxicity was published[18] and a year later another study was available for progression-free survival and overall survival.[19] We did not modify our initial SNP selection on the basis of these reports; however, we used preliminary data from a pazopanib pharmacogenetics study to add the *IL8* SNP.[8]

Interpretation

Our study is the first prospective evaluation of SNPs as predictors of efficacy and toxicity of first-line sunitinib in previously untreated patients with advanced clear-cell renal-cell carcinoma. We noted a strong association between two missense *VEGFR3* polymorphisms and progression-free survival after treatments with sunitinib. We also show that the *CYP3A5*1* allele is associated with an increased risk of sunitinib dose reductions due to toxic effects. If our findings are confirmed in an independent series, these polymorphisms could be used to identify subsets of patients that could benefit from alternative treatment options.

Contributors

JG-D and CR-A designed the study. JG-D, LJL-G, and CR-A did the data collection. JG-D, LJL-G, MR, and CR-A did the data analysis, data interpretation, and wrote the manuscript. JG-D, EE, DEC, AGA, MAC, JAA, EG, JP, JB, BM, EM, FM, and AF provided study materials or recruited patients. All authors critically reviewed the manuscript and approved the final version.

Conflicts of interest

JG-D and JB have received consultancy and advisory board fees from Pfizer. All other authors declare that they have no conflicts of interest.

Acknowledgments

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Supplementary Table 1. Most common sunitinib toxicities developed by the patients and clinical consequences in terms of dose reductions.

Event	Any grade n (%)	Grade 1 n (%)	Grade 2 n (%)	Grade 3 n (%)
Asthenia	67 (71)	26 (27)	31 (33)	10 (10)
Mucositis	64 (67)	26 (27)	31 (33)	7 (7)
Diarrhea	49 (52)	25 (26)	20 (21)	4 (4)
Neutropenia	40 (42)	19 (20)	15 (16)	6 (6)
Hand foot syndrome	39 (41)	12 (13)	21 (22)	6 (6)
Hypertension	33 (35)	9 (9)	21 (22)	3 (3)
Thrombocytopenia	26 (27)	16 (17)	6 (6)	4 (4)
Anemia	24 (25)	14 (15)	8 (8)	2 (2)
Hypothyroidism	20 (21)	10 (10)	10 (10)	0 (0)
Elevated creatinine	22 (23)	14 (15)	7 (7)	1 (1)
Edema	12 (13)	10 (10)	2 (2)	0 (0)
Leukopenia	9 (9)	4 (4)	5 (5)	0 (0)
Dose red.[a]	47 (49)	-	-	-
Dose red.[a] 50mg initial dose	44 (52)	-	-	-

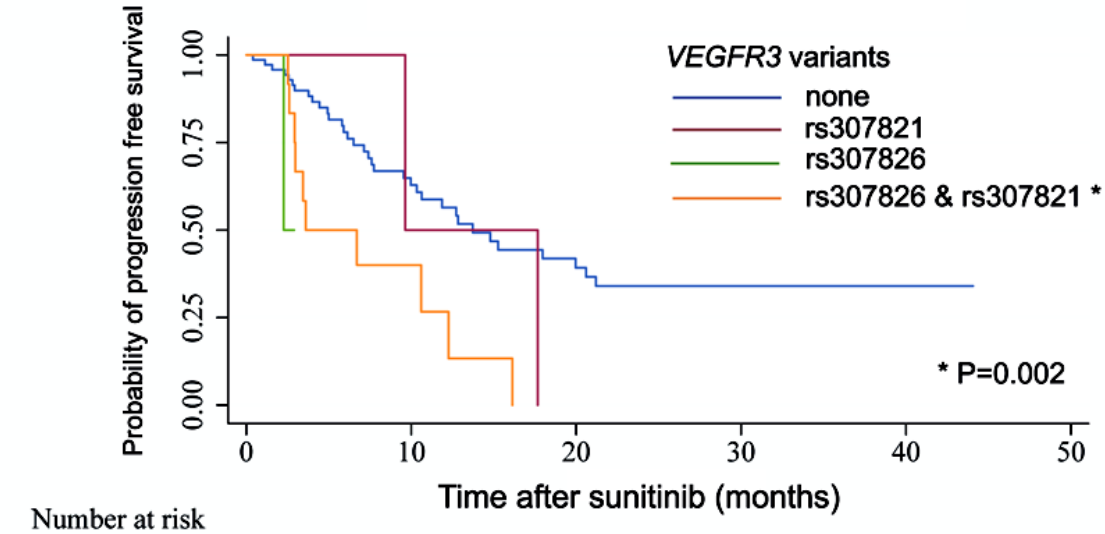
[a] Dose red. stands for sunitinib dose reduction due to toxicity and indicates the number of patients with this event.
[b] 50 mg initial dose, indicates the subgroup of patients that started sunitinib treatment with 50 mg and required dose reduction.

Supplementary Table 2. Toxicities associated with sunitinib dose reductions.

Toxicities[a]	All patients			Patients with 50 mg initial dose		
	HR	95% CI	*P* value	HR	95% CI	*P* value
Hand foot syndrome	6·12	2·46-15·2	**0·000098**	7·73	2·86-20·9	**0·000053**
Any toxicity grade 3	5·94	2·42-14·6	**0·00010**	6·66	2·53-17·5	**0·00017**
Mucositis	5·71	2·14-15·3	**0·00051**	3·91	1·40-10·9	**0·0053**
Any toxicity grade ≥2 1st cycle	3·94	1·50-10·3	**0·0053**	4·54	1·57-13·2	**0·0090**
Thrombocytopenia	3·97	1·48-10·7	**0·0063**	4·00	1·30-12·3	**0·015**
Anemia	2·83	1·08-7·43	**0·034**	2·93	1·01-8·53	**0·049**
Neutropenia	2·09	0·91-4·78	0·082	2·56	1·04-6·28	**0·041**

[a] Only toxicities associated with dose reductions with P values < 0·05 are shown.

Supplementary Figure 1. PFS of renal cancer patients treated with sunitinib stratified according to *VEGFR3* rs307826 and rs307821 polymorphisms. Because the SNPs showing the strongest association with PFS, rs307826 and rs307821, were in moderate LD (r^2= 0.55) we performed additional analysis trying to determine which could be the functional variant. For that purpose patients were grouped according to rs307826 and rs307821 genotypes and compared using the Kaplan-Meier method (72 patients carried simultaneously wild type alleles for rs307826 and rs307821, 13 patients were heterozygous for both polymorphisms, 2 patients were heterozygous for rs307821 and carried wild type alleles for rs307826 and 2 patients were heterozygous for rs307826 and carried wild type alleles for rs307821). Multivariable analysis comparing patients with no variant alleles and those heterozygous for both SNPs gave an HR of 3·1 (95%CI=1·5-6·5, P=0·0023), unadjusted P value from the log-rank test 0·0020. The other groups were not significantly different.



Number at risk

none	72	31	15	2	1	0
rs307821	2	1	0	0	0	0
rs307826	2	0	0	0	0	0
rs307826 & rs307821	13	4	0	0	0	0

Supplementary Table 3: Clinical factors and association with response, progression-free survival, and overall survival in patients with renal cell carcinoma treated with sunitinib

	Progressive disease as best response		**Progression-free survival**		**Overall survival**	
	Hazard ratio (95% CI)	p	Hazard ratio (95% CI)	p	Hazard ratio (95% CI)	p
MSKCC risk factors*	4·14 (1·30–13·2)	0·016	1·64 (0·96–2·81)	0·072	2·21 (1·00–4·41)	0·025
Sex	3·66 (1·37–11·7)	0·030	1·70 (0·91–3·16)	0·094	2·80 (1·30–6·03)	0·009

*According to the number of Memorial Sloan-Kettering Cancer Center (MSKCC) prognostic factors; patients were classified into favourable, intermediate, and poor prognosis groups.

Supplementary Table 4: Clinical factors and association with dose reductions and toxic effects

	Hazard ratio (95% CI)	p
Dose reduction due to toxic effects		
Sex	2·6 (1·3–5·4)	0·005
Hypertension		
Hand-foot syndrome		
MSKCC risk group	0·48 (0·2–1·0)	0·063
xxx		
Mucositis		

ARTÍCULO 2: Prospective study assessing hypoxia-related proteins as markers for the outcome of treatment with sunitinib in advanced clear-cell renal cell carcinoma.

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Resumen: Trabajos previos han sugerido que la expresión de marcadores de hipoxia en el tejido tumoral puede estar asociada con respuesta a fármacos antiangiogénicos. En consecuencia, nos propusimos estudiar la capacidad de dichos marcadores para predecir la respuesta a sunitinib en carcinoma renal de células claras.
Métodos: La expresión de 8 proteínas clave relacionadas con la hipoxia (CAIX, HIF1A, HIF2A, VEGFA, VEGFR1, VEGFR2, VEGFR3 y PDGFRB) y la P-glicoproteína fue determinada mediante inmunohistoquímica en 67 tumores primarios de cáncer renal de células claras de una serie de pacientes reclutados de forma prospectiva y tratados con sunitinib en primera línea. Los niveles de expresión, la presencia de inactivación del gen VHL y el contenido de RNA mensajero del gen EGLN3 fueron comparados entre casos respondedores y no respondedores.
Resultados: Los niveles altos de expresión de HIF2A y PDGFRB se asociaron de una forma estadísticamente significativa con la probabilidad de alcanzar una respuesta objetiva a sunitinib según criterios RECIST (P = 0.024 y P = 0.026; respectivamente); además, una expresión elevada de VEGFR3 se asoció con una supervivencia libre de progresión mayor (P = 0.012) y dicha sobreexpresión se correlacionó de forma negativa con la presencia del polimorfismo rs307826 en dicho gen (P = 0.002). Este polimorfismo había sido identificado por nuestro grupo como un predictor de resistencia al fármaco en trabajos previos. Con respecto a la supervivencia global, la alta expresión de VEGFA estuvo asociada con una supervivencia peor (P = 0.009) y la de HIF2A con una supervivencia mayor (P = 0.048). Así mismo, niveles altos de mRNA del gen

EGLN3 se asociaron con una SG más corta (P = 0.023).

Conclusión: Nuestro trabajo ha establecido una asociación entre la expresión de varias proteínas implicadas en hipoxia y la eficacia de sunitinib. Además, una baja expresión de VEGFR3 se asoció con unos resultados peores y con la presencia del alelo rs307826 en dicho gen, reforzando su potencial uso como marcador de resistencia a sunitinib.

Aportación personal: Jesús García-Donas fue el promotor del trabajo, participando en su concepción y diseño. Colaboró en el análisis e interpretación de los resultados así como en la elaboración y presentación para publicación del manuscrito final

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Prospective study assessing hypoxia-related proteins as markers for the outcome of treatment with sunitinib in advanced clear-cell renal cell carcinoma

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: Previous studies suggest that expression of hypoxia markers may be associated with response to antiangiogenic drugs. Thus, we aimed to identify predictors of sunitinib outcome in clear-cell renal cell carcinoma (ccRCC).

Patients and methods: The expression of eight key proteins related to hypoxia (CAIX, HIF1A, HIF2A, VEGFA, VEGFR1, VEGFR2, VEGFR3 and PDGFRB) and P-glycoprotein were assessed by immunohistochemistry in 67 primary ccRCC samples from prospectively recruited patients treated with first-line sunitinib. The proteins expression, VHL inactivation and EGLN3 mRNA content were compared with the patients' response to sunitinib.

Results: High expression of HIF2A and PDGFRB was associated with better sunitinib RECIST objective response (P = 0.024 and P = 0.026; respectively) and increased VEGFR3 expression was associated with longer progression-free survival (P = 0.012). VEGFR3 overexpression showed a negative correlation with VEGFR3 polymorphism rs307826 (P = 0.002), a sunitinib resistance predictor. With respect to overall survival (OS), high VEGFA was associated with short (P = 0.009) and HIF2A with long (P = 0.048) survival times. High EGLN3 mRNA content was associated with shorter OS (P = 0.023).

Conclusions: We found an association between several proteins involved in hypoxia and sunitinib efficacy. In addition, low VEGFR3 expression was associated with worse outcome and with VEGFR3 rs307826 variant allele, reinforcing VEGFR3 as a marker of sunitinib resistance.

Key words: renal carcinoma, EGLN3, hypoxia, immunohistochemistry, predictive marker, sunitinib

introduction

More than 260 000 new renal cancer cases are diagnosed around the world every year and kidney cancer incidence seems to be rising ~2%–3% per decade [1]. Advanced disease remains a lethal condition, but the development of new-targeted therapies, such as VEGF/VEGFR and mTOR inhibitors, has dramatically improved survival and daily management [2]. In fact, now that several therapeutic options are available, attending physicians face the challenge of selecting drugs to treat kidney cancer patients without reliable predictors of response that could help prioritizing one drug over another. Currently, this selection relies on personal experience and the anticipated toxic effect profile of the drug, without support from molecular characteristics of the tumor and the patient, which could be critical for drug

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outcome. Therefore, predictors of efficacy for targeted treatments in kidney cancer are urgently needed.

Sunitinib (Sutent; Pfizer, New York, NY) is an approved tyrosine kinase inhibitor (TKI) of VEGFR1-3, PDGFR, KIT, FLT3 and CSF-1, and is widely used for first-line treatment of advanced clear-cell renal cell carcinoma (ccRCC) [2]. Recently, several studies have investigated single-nucleotide polymorphisms [3], plasma factors and circulating endothelial cells [4, 5] as potential markers of response to antiangiogenic treatment in renal cancer with promising results. With respect to molecular events and expression of proteins related to tumor hypoxia, a crucial mechanism for renal cancer development and antiangiogenic drug therapy, some studies have investigated the association between Von Hippel–Lindau (VHL) inactivation and antiangiogenic drug response, mainly obtaining negative results [6–10]. For Carbonic Anhydrase IX (CAIX) expression, no clear conclusions have been reached [11, 12], and for hypoxia-inducible factors (HIFs), a recent study suggested that low HIF1A expression might be associated with better progression-free survival (PFS) of patients treated with sunitinib [9], while high HIF2A expression has been suggested to improve response to sunitinib [12]. However, the tumor mRNA expression of prolyl hydroxylase EGLN3, an excellent marker of hypoxia in RCC [13], has not been investigated in relation to response to antiangiogenic drugs. Assessment of these markers in prospective studies is a crucial step to determine their predictive value and to facilitate their integration into the clinic. In this exploratory study, we aimed to identify new biomarkers of sunitinib efficacy and to validate others previously reported, focusing on hypoxia-related proteins using 71 ccRCC samples collected in an observational prospective study [14].

patients and methods

study population

The current work is a pre-planned observational prospective study aimed at the identification of molecular tumor markers of sunitinib efficacy in ccRCC, carried out in a population that was reported in an earlier study [14]. In this previous study, 101 patients with RCC treated with first-line sunitinib were included to investigate the association between SNPs and sunitinib efficacy and toxic effect. Drug schedule, dose reduction policy, and timing of radiological assessments were up to the discretion of the individual physicians, in accordance with current local practice guidelines. The present study used a subset of 71 consecutive patients of whom formalin-fixed paraffin-embedded tumor material was available.

immunohistochemical study

The detection of CAIX, HIF1A, HIF2A, VHL, VEGFA, VEGFR1, VEGFR2, VEGFR3, PDGFRB and P-glycoprotein (Pgp) was carried out by immunohistochemistry (IHC) in tissue microarrays (supplementary Methods, available at *Annals of Oncology* online) using specific antibodies (supplementary Table S1, available at *Annals of Oncology* online). The IHC scoring was based on the average percentage of cells with positive staining, as in the Human Protein Atlas (http://www.proteinatlas.org): 0%–5%: absent (0), 5%–25%: weak (1), 25%–75%: moderate (2) and >75%: strong (3). Supplementary Table S2, available at *Annals of Oncology* online shows the staining score for each protein marker analyzed and the number of tumors included in each category.

assessment of *VHL* inactivation

VHL mutations were detected through PCR (supplementary Methods, available at *Annals of Oncology* online), and tumors harboring *VHL* frameshifts, stop gains, stop losses, splicing defects, mutations previously described as pathogenic and mutations in codons with previously described pathogenic mutations, were considered pathogenic ($n = 17$, supplementary Table S3, available at *Annals of Oncology* online). In addition, c.338G>A (R113Q) was considered pathogenic based on Polyphen2 predictions (http://genetics.bwh.harvard.edu/pph2/). Cases carrying a pathogenic *VHL* mutation and cases without VHL protein expression were classified as *VHL* inactivated ($n = 20$). Only tumors successfully sequenced for all three exons, with a wild type *VHL* sequence and a positive staining of VHL protein were considered to have a non-altered VHL function ($n = 11$); otherwise, VHL inactivation status was defined as non-assessable.

EGLN3 mRNA quantification

The mRNA content of *EGLN3* was quantified through qRT-PCR using specific primers and probes (supplementary Methods, available at *Annals of Oncology* online). Normalization was carried out with the internal standard β-glucuronidase and the ΔΔCt method was used for the calculation of mRNA content. In 65 of the 67 primary ccRCC samples, *EGLN3* mRNA was successfully quantified.

statistical analyses

This study defined objective response according to RECIST criteria, and PFS and overall survival (OS) as previously described [14]. Protein expression and VHL inactivation status were tested as dichotomous variables and *EGLN3* mRNA content was recorded as a continuous variable. These variables were tested against progression of the disease (PD) as best objective response using logistic regression, and against PFS and OS using Cox regression. Proteins with a $P < 0.1$ in the univariate analyses were selected for multivariable analyses, including as covariates the MSKCC prognostic classification and gender [14]. Given the explorative nature of this study, the *P*-values were not corrected for multiple testing. The correlation between the expression of the different proteins, VHL status and *EGLN3* mRNA content was evaluated using the Spearman test. Bilateral *P*-values <0.05 were considered significant. SPSS version 19.0 was used for statistical analysis.

results

study population

Of the initial 71 patients that had undergone nephrectomy, four were excluded from the analysis (two corresponded to primary RCCs with a histology different from ccRCC and two to patients with no primary tumor material available, only metastases). The main characteristics of the 67 patients included in the analysis are presented in Table 1. The median PFS of the patients after sunitinib treatment was 12.7 months and 46 (69%) were alive at the time of the analysis. Objective response was assessed in 60 patients with measurable disease: 31 (52%) had partial response, 20 (33%) had stabilization of the disease and 9 (15%) had PD. Patients with high MSKCC risk factor scores had worse OS (HR = 2.2, 95% confidence interval (CI) 1.0–4.7, $P = 0.045$) and showed a tendency of higher risk of PD as best response ($P = 0.066$). Women showed a trend for higher PD risk ($P = 0.078$). These clinical characteristics are similar to those described previously for the whole series [14].

Table 1. Patient and clinical characteristics

Characteristic	No.	%
Age at sunitinib (years)	66	
Min–max (IQR[a])	46–82 (55–72)	
Sex		
Male	45	67
Female	22	33
ECOG score		
0	19	28
1	37	55
2	6	9
Unknown	5	7
No. of metastatic sites		
1	17	25
2	35	52
3	11	16
4	4	6
Common metastasis sites		
Lung	49	73
Lymph nodes	34	51
Bone	16	24
Kidney	12	18
Liver	9	13
MSKCC risk factors[b]		
0 (favorable)	36	54
1–2 (intermediate)	30	45
≥3 (poor)	1	1

[a]Interquartile range.

[b]Risk groups according to Memorial Sloan-Kettering Cancer Center (MSKCC) prognostic factors: ECOG performance status >1, high LDH levels (>1.5 times upper limit of normal), low serum hemoglobin, high corrected serum calcium (>10 mg/dl) and no nephrectomy.

proteins involved in hypoxia and angiogenesis are associated with outcome of sunitinib treatment

We compared the expression of nine different proteins related to hypoxia, angiogenesis or sunitinib transport in the tumors. As shown in supplementary Table S2, available at *Annals of Oncology* online, there was a substantial variability in the expression of these proteins in the primary ccRCC tumors and the expression of several of these proteins was correlated. The strongest correlation was between VEGFA and VEGFR2 ($r = 0.60$ and $P = 2 \times 10^{-7}$, supplementary Table S4, available at *Annals of Oncology* online).

When we studied the association between the expression of specific proteins and sunitinib response, we found that high expression of HIF2A and PDGFRB was associated with protection against PD [odds ratio (OR) = 0.11, 95% CI 0.02–0.75, $P = 0.024$ and OR = 0.04, 95% CI 0.002–0.68, $P = 0.026$, respectively, Table 2]. With respect to PFS, high VEGFR3 expression was associated with longer times to progression (HR = 0.40, 95% CI 0.20–0.82, $P = 0.012$; Figure 1). Regarding OS, high VEGFA expression was associated with short survival times (HR = 4.29, 95% CI 1.43–12.8, $P = 0.0092$; Figure 1), and high HIF2A expression with long survival times (HR = 0.39, 95% CI 0.15–0.99, $P = 0.048$; Figure 1).

Interestingly, we had previously found that the missense variants of *VEGFR3* rs307826 and rs307821, in high linkage disequilibrium, were associated with short PFS in sunitinib-treated patients [14]. When we compared VEGFR3 protein expression with rs307826 and rs307821, we found a significant correlation, with the variant alleles showing a significantly lower expression of VEGFR3 protein ($r = -0.38$, $P = 0.0019$ and $r = -0.32$, $P = 0.011$, respectively, Figure 2). Other polymorphisms in *VEGFA* (rs699947, rs2010963, rs1570360) and *VEGFR2* (rs699947, rs2010963, rs1570360) did not show a correlation with the expression of their respective proteins.

Table 2. Proteins associated with sunitinib efficacy in ccRCC

Protein	Univariate analyses[a]			Multivariable analyses[b]		
	HR[c]	95% CI	*P*-value	HR[c]	95% CI	*P*-value
PD as best response						
HIF2A	0.20	0.04–0.91	**0.037**	0.11	0.017–0.75	**0.024**
PDGFRB	0.13	0.014–1.08	0.059	0.04	0.002–0.68	**0.026**
Pgp	8.00	0.93–69.2	0.059	7.08	0.76–66.3	0.086
PFS						
VEGFR3	0.39	0.20–0.80	**0.0093**	0.403	0.20–0.82	**0.012**
VEGFA	2.56	1.04–6.29	**0.041**	2.560	0.99–6.63	0.053
HIF2A	0.52	0.26–1.04	0.064	0.537	0.26–1.13	0.100
OS						
VEGFA	3.58	1.38–9.33	**0.0089**	4.29	1.43–12.8	**0.0092**
HIF2A	0.34	0.14–0.83	**0.017**	0.39	0.15–0.99	**0.048**
VEGFR2	2.14	0.90–5.09	0.086	2.05	0.85–4.91	0.108
Pgp	2.41	0.88–6.57	0.087	2.21	0.81–6.07	0.123

P-values <0.05 are shown in bold.

[a]Only proteins with $P < 0.1$ are presented; proteins with $P < 0.1$ in the univariate analyses were selected for multivariable analyses.

[b]Multivariable analyses included as covariates the MSKCC prognostic classification and gender.

[c]HR <1.0 indicates that high expression of the protein associates with better outcome, HR >1.0 indicates association with worse outcome.

EGLN3 mRNA content is associated with the overall survival of the patients

VHL inactivation and hypoxia, which in RCC correlate with *EGLN3* mRNA content [13], could be relevant markers of response to antiangiogenic drugs. In fact, we found that *EGLN3* mRNA content, quantified in 65 cases, showed a positive correlation with CAIX ($r = 0.44$, $P = 0.0007$) and with *VHL* inactivation ($r = 0.42$, $P = 0.02$; supplementary Table S4, available at *Annals of Oncology* online). In addition, *EGLN3* mRNA content was negatively correlated with the expression of PDGFRB ($r = -0.46$, $P = 0.0002$).

To determine whether VHL inactivation and *EGLN3* mRNA content were associated with PD as best response, PFS and OS, we carried out a multivariable analysis. We did not find an association between *VHL* inactivation and sunitinib efficacy, but we found that high *EGLN3* mRNA content was associated with a shorter OS ($P = 0.023$). When the RCCs were divided into high and low *EGLN3* expression groups, using the mean expression as the cutoff value rather than using *EGLN3* mRNA as a continuous variable, cases with high expression had an HR of 2.89 (95% CI 1.04–8.05, $P = 0.042$, data not shown).

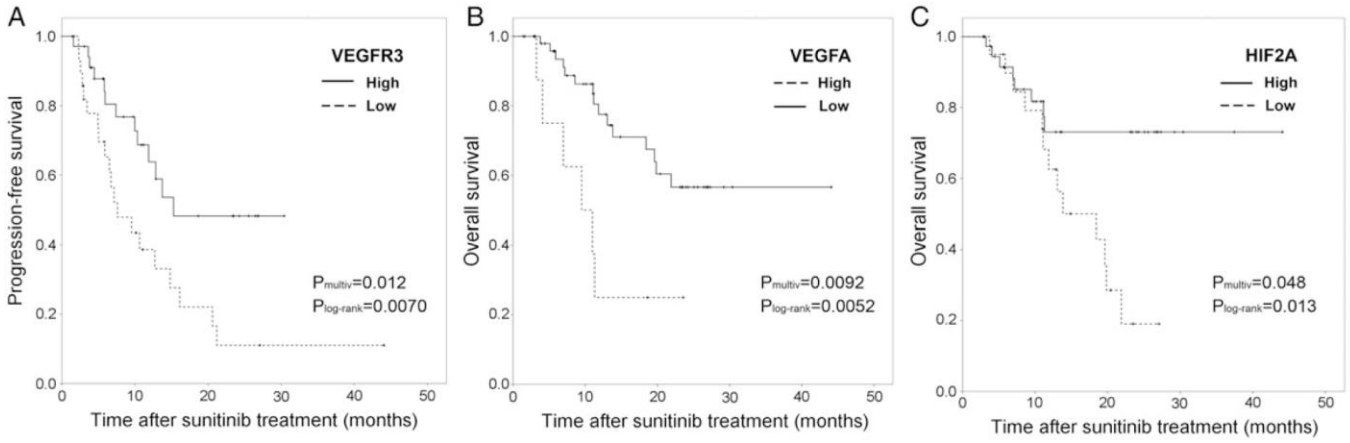

Figure 1. Progression-free survival and overall survival in ccRCC patients after treatment with sunitinib. (A) Progression-free survival of patients grouped according to VEGFR3 protein expression; median progression-free survival for patients with tumors with low and high VEGFR3 expression was 7.6 and 15.3 months, respectively. (B) Overall survival of patients grouped according to VEGFA protein expression; median overall survival for patients with tumors with low VEGFA expression was not reached and for patients with tumors with high VEGFA expression it was 9.5 months. (C) Overall survival of patients grouped according to HIF2A protein expression; median overall survival for patients with tumors with low HIF2A expression was 18.4 months and, for patients with tumors with high HIF2A, it was not reached. *P*-values are from multivariable Cox regression analysis and from the univariate log-rank test.

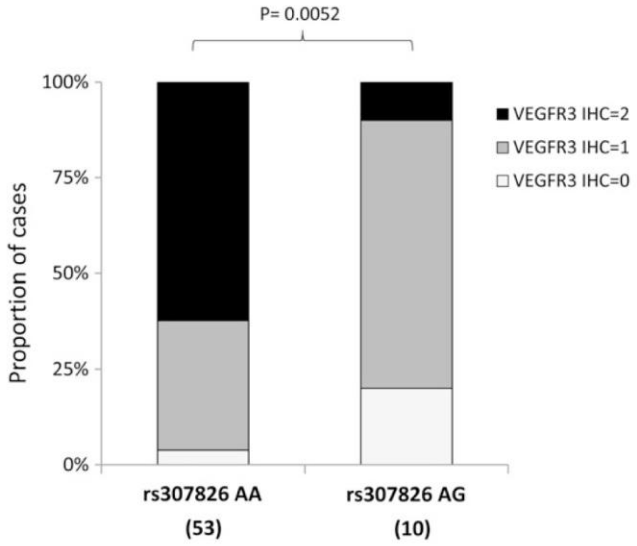

Figure 2. Immunohistochemical staining of VEGFR3 protein according to *VEGFR3* rs307826 genotype. Of the 53 cases with the rs307826 AA genotype, 2 (4%), 18 (34%) and 33 (62%) had a VEGFR3 staining of 0, 1 and 2, respectively. Of the 10 cases with the rs307826 AG genotype, 2 (20%), 7 (70%) and 1 (10%) had a VEGFR3 staining of 0, 1 and 2, respectively. The *P*-value shown was calculated by the χ^2 test.

discussion

In this study, we focused on the identification of tumor markers of response to sunitinib, by using a prospective series of patients receiving sunitinib for advanced ccRCC as first-line treatment [14]. We found that *EGLN3* mRNA content and VEGFA expression were associated with worse outcome, while overexpression of HIF2A, PDGFRB and VEGFR3 were associated with better response and survival.

VEGFR3 is a transmembrane tyrosine kinase receptor traditionally associated with lymphangiogenesis; however, recent studies have shown expression of VEGFR3 in the tumor blood vasculature [15]. VEGFR3 has been proposed as a pharmacodynamic marker for sunitinib response [16, 17]. In addition, we previously found that VEGFR3 was associated with worse outcome of sunitinib treatment through two missense polymorphisms in high linkage disequilibrium (rs307826, T494A; rs307821, R1324L) [14]. In the present study, we found a large variability in VEGFR3 expression in tumors and a statistically significant association between a high tumor expression of VEGFR3 protein and longer PFS of the patients treated with sunitinib ($P = 0.012$, Table 2). Interestingly, we also found a strong correlation between VEGFR3 protein expression in the tumor and rs307826 and rs307821 *VEGFR3* variant alleles. The mechanisms by which these polymorphisms exert their effect is unknown; however, these results may imply a low stability of the variant VEGFR3 protein, which in turn could lead to decreased sunitinib effects.

VEGFA has been studied in relation with sunitinib treatment as a dynamic marker [17, 18] and SNPs in *VEGFA* have been evaluated as predictors of sunitinib response [14, 19]; however, the results obtained are inconclusive. Regarding its expression, a study suggested a better outcome in tumors with high VEGFA content [20], but our study showed a significant association between high VEGFA expression and worse OS ($P = 0.0092$, Table 2), similar to other studies investigating RCC prognostic factors [21, 22].

HIFs are oxygen-sensitive transcription factors, which regulate biological processes that promote adaptation to oxygen deprivation. In our study, overexpression of HIF2A was significantly associated with clinical benefit, longer OS and a tendency for longer PFS of the patients (Table 2). In agreement, there are some studies that have associated high HIF2A levels with improved response to sunitinib [12]. Concerning VHL, a key regulator of the hypoxia response pathway and the gene most frequently mutated in ccRCC, our study did not find an association between VHL inactivation and sunitinib efficacy, in

line with previous works [7, 23]. However, the number of samples evaluated was relatively small ($n = 31$). To get an accurate assessment of hypoxia in the tumors, we quantified the mRNA content of *EGLN3* [13], and found a positive correlation with CAIX ($P = 0.00072$) and VHL inactivation ($P = 0.02$). Our data support previous studies in which CAIX failed to predict response and survival in ccRCC [9, 11, 12]; however, we found that high *EGLN3* mRNA content was associated with short OS ($P = 0.023$). This suggests that highly hypoxic tumors, although might have a better initial response to antiangiogenic drugs, could have a worse prognosis, as evidenced by the shorter OS found for tumors with high VEGFA or high *EGLN3* expression.

Some limitations of this study are caused by intrinsic characteristics of the techniques used, such as IHC scoring and *EGLN3* mRNA measurement, which can vary among institutions since these determinations are not used in daily practice and no consensus criteria for quantification have been established yet. However, the IHC scoring was carried out independently by two pathologists and *EGLN3* expression was measured using a quantitative technique. Other caveats are related to the observational nature of the study, which cannot distinguish among predictive and prognostic markers, but can only suggest associations. In addition, subsequent anticancer treatments, that could potentially impact OS, were not recorded. There is also the need to validate the results of this exploratory study in an independent series of patients, to precisely determine the predictive value of the proposed markers.

In conclusion, we have confirmed HIF2A and VEGFR3 as potential predictors of sunitinib efficacy in ccRCC, and found an association between high VEGFA expression and shorter OS. In addition, significant correlations between rs307826 and rs307821 and VEGFR3 protein content was identified for the first time, suggesting a possible biological mechanism for the worse outcome of sunitinib treatment associated with these variant alleles. Finally, a new marker of ccRCC hypoxia, *EGLN3* expression, was associated with short OS. If these finding are validated in independent series, prospective interventional trials based on molecular biomarkers and aiming at the identification of new therapeutic strategies for ccRCC should be undertaken to ultimately improve RCC outcome.

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disclosure

JG-D and JB receive consultant and advisory board fees from Pfizer. The other authors declare that they have no conflicts of interest.

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MATERIAL SUPLEMENTARIO

SUPPLEMENTARY METHODS

Tissue microarrays (TMAs) construction and immunohistochemical study

Hematoxylin and eosin stained sections of each FFPE tumor sample were examined by two pathologists to confirm the diagnosis and to select areas representative of each tumor to construct TMAs. Two TMAs containing all tumor samples were constructed with 2 cores of each tumor placed at different positions in the TMA.

Two pathologists (M.M. and I.A.) evaluated independently and blinded regarding clinical data, the intensity and extension of the staining for all antibodies. Discrepancies were scarce (<10% of studied tissue cores) and resolved jointly by consensus.

To define immunopositive staining we used as cutoff the median cases observed for each IHC category, so a balanced number of cases showed negative and positive staining. The only exception was VEGFA, were strong and moderate expression were grouped together, similarly to previous reports [1].

Assessment of VHL inactivation

To detect VHL mutations, tumor areas of each FFPE sample were extracted, avoiding non-tumor tissue regions, and used for genomic DNA isolation using the DNeasy Blood and Tissue kit (Qiagen). The coding region of VHL was amplified in three independent PCR reactions as described previously [2]. The PCR amplification products were purified using the PCR Purification Kit (Qiagen) and run on an ABI PRISM 3700 DNA Analyzer capillary sequencer (Applied Biosystems).

There were 5 cases in which VHL protein was not detected, 2 of which corresponded to cases with a pathogenic VHL mutation (a deletion of 17 nucleotides, c.308_324delCTGGCACGGGCCGCCGG, and an amino acid substitution, c.233A>G).

EGLN3 mRNA quantification through quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

Total RNA was extracted from FFPE tumor samples using the RNeasy FFPE kit (Qiagen) and the concentration was determined by using NanoDrop 1000 (Thermo Scientific). One microgram of total RNA was reverse transcribed using Superscript II (Invitrogen) and random primers following the manufacturer's instructions. The mRNA content of *EGLN3* was quantified by qRT-PCR with the Sequence Detection System 7900HT (Applied Biosystems) using specific primers and probes from the Universal ProbeLibrary Set, Human (Roche), available upon request. Negative controls were included in all PCR series, and assays were carried out in triplicate.

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SUPPLEMENTARY TABLES

Supplementary Table 1. List of antibodies and conditions used for IHC.

Supplementary Table 2. Expression of the nine proteins analyzed in the primary ccRCCs.

Supplementary Table 3. Pathogenic *VHL* mutations found in 17 ccRCC cases.

Supplementary Table 4. Correlation between the expression of nine proteins, *EGLN3* mRNA content and VHL status in primary ccRCC.

Supplementary Table 1. List of antibodies and conditions used for IHC.

Antibody	Clone	Supplier	Reference	Dilution	Visualization system & immunostainer
CAIX	Polyclonal	Abcam	AB15086	1:300	EnVision Flex Autostainer
HIF1A	EP1215Y	Abcam	AB51608	1:75	EnVision Flex Autostainer
HIF2A	Polyclonal	Novus Biologicals	NB100-122	1:200	EnVision Flex Autostainer
VHL	IG33	Thermo	MS-690-P	1:50	Vision Biosystem Bond
VEGFA	SP28	Abcam	AB27620	1:1	Vision Biosystem Bond
VEGFR1	Polyclonal	Sigma-Aldrich	HPA014290	1:50	EnVision Flex Autostainer
VEGRF2	A-3	Santa Cruz Biotechnology	sc-6251	1:100	EnVision Flex Autostainer
VEGFR3	9D9F9	Millipore	MAB3757	1:200	EnVision Flex Autostainer
PDGFRB	2B3	Cell Signaling Technology	3175	1:100	EnVision Flex Autostainer
Pgp	C494	Dako	M3522	1:200	EnVision Flex Autostainer

Supplementary Table 2. Expression of the nine proteins analyzed in the primary ccRCCs.

Protein	Positive cases[a] n (%)	Absent (0) n (%)	Weak (1) n (%)	Moderate (2) n (%)	Strong (3) n (%)
CAIX	47 (81)	3 (5)	3 (5)	5 (9)	47 (81)
HIF1A	25 (42)	35 (58)	21 (35)	4 (7)	-
HIF2A	39 (65)	4 (7)	17 (28)	34 (57)	5 (8)
VEGFA	8 (13)	31 (50)	23 (37)	7 (11)	1 (2)
VEGFR1	21 (33)	43 (67)	18 (28)	3 (5)	-
VEGRF2	19 (30)	45 (70)	17 (27)	2 (3)	-
VEGFR3	34 (53)	4 (6)	26 (41)	34 (53)	-
PDGFRB	31 (50)	31 (50)	25 (40)	6 (10)	-
Pgp	34 (57)	6 (10)	20 (33)	25 (42)	9 (15)

[a] Grey background indicates IHC scores regarded as immunopositive.

Supplementary Table 3. Pathogenic *VHL* mutations found in 17 ccRCC cases.

Pathogenic *VHL* mutation	**Effect**
c.174_181delCCGCGGCCinsA	Frameshift [a]
c.209_226delAGCCCTCCCAGGTCATCT	p.E70_F76delinV [b]
c.214T>C	S72P [c]
c.233A>G	N78S [c]
c.302_308delTGCCGCCinsCGCG	p.L101_P103delinsPR [b]
c.307_309delCCT	p.P103del [b]
c.308_324delCTGGCACGGGCCGCCGG	Frameshift [a]
c.338G>A	R113Q [d]
c.341-2A>G	Splicing defect [a]
c.405delA	Frameshift [a]
c.449_456delATATCACA	Frameshift [a]
c.463+1G>A	Splicing defect [a]
c.465C>G/ c.471A>G	Y175X [a] / R177R
c.491A>T	Q164L [b]
c.505C>G/ c.175_190delCCGCGGCCCGTGCTGC	L169V/ frameshift [a]
c.579insA	Frameshift [a]
c.641insT	Stop loss [a]

[a] Frameshifts, splicing defects, stop gains and stop losses were considered pathogenic.
[b] Mutations in codons with previously described pathogenic mutations were considered pathogenic.
[c] Mutations previously described as pathogenic.
[d] Considered pathogenic based on Polyphen2 predictions

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Supplementary Table 4. Correlation between the expression of nine proteins, *EGLN3* mRNA content and VHL status in primary ccRCC.

	CAIX	**HIF1A**	**HIF2A**	**VEGFA**	**VEGFR1**	**VEGFR2**	**VEGFR3**	**PDGFRB**	**Pgp**	***VHL* inactivation**	***EGLN3* mRNA**
CAIX		**r= -0.29 P= 0.028**	r= -0.07 P= 0.58	r= -0.18 P= 0.17	r= -0.04 P= 0.78	r= -0.14 P= 0.30	r= -0.01 P= 0.94	r= -0.21 P= 0.12	r= -0.17 P= 0.20	r= 0.24 P= 0.23	**r= 0.44 P= 0.00072**
HIF1A	**r= -0.29 P= 0.028**		r= 0.19 P= 0.14	**r= 0.29 P= 0.028**	r= 0.22 P= 0.09	r= 0.10 P= 0.44	r= 0.11 P= 0.39	**r= 0.27 P= 0.041**	r= -0.01 P= 0.93	**r= -0.49 P= 0.011**	r= -0.12 P= 0.36
HIF2A	r= -0.07 P= 0.58	r= 0.19 P= 0.14		r= 0.14 P= 0.30	**r= 0.27 P= 0.034**	r= 0.05 P= 0.72	r= 0.22 P= 0.09	**r= 0.27 P= 0.039**	r= -0.15 P= 0.26	r= -0.02 P= 0.92	r= -0.08 P= 0.53
VEGFA	r= -0.18 P= 0.17	**r= 0.29 P= 0.028**	r= 0.14 P= 0.30		r= 0.04 P= 0.74	**r= 0.60 P= 0.00000023**	r= 0.07 P= 0.58	r= 0.00 P= 1.00	r= 0.19 P= 0.15	r= 0.04 P= 0.83	r= -0.19 P= 0.15
VEGFR1	r= -0.04 P= 0.78	r= 0.22 P= 0.09	**r= 0.27 P= 0.034**	r= 0.04 P= 0.74		r= -0.16 P= 0.20	r= -0.14 P= 0.26	r= 0.00 P= 1.00	r= -0.06 P= 0.67	r= -0.18 P= 0.36	r= -0.07 P= 0.60
VEGFR2	r= -0.14 P= 0.30	r= 0.10 P= 0.44	r= 0.05 P= 0.72	**r= 0.60 P= 0.00000023**	r= -0.16 P= 0.20		r= 0.13 P= 0.30	r= -0.14 P= 0.27	r= 0.22 P= 0.09	r= -0.07 P= 0.71	r= -0.16 P= 0.22
VEGFR3	r= -0.01 P= 0.94	r= 0.11 P= 0.39	r= 0.22 P= 0.09	r= 0.07 P= 0.58	r= -0.14 P= 0.26	r= 0.13 P= 0.30		r= 0.03 P= 0.80	r= -0.14 P= 0.27	r= 0.07 P= 0.71	r= 0.00 P= 0.98
PDGFRB	r= -0.21 P= 0.12	**r= 0.27 P= 0.041**	**r= 0.27 P= 0.039**	r= 0.00 P= 1.00	r= 0.00 P= 1.00	r= -0.14 P= 0.27	r= 0.03 P= 0.80		**r= -0.34 P= 0.0090**	r= 0.07 P= 0.74	**r= -0.46 P= 0.00019**
Pgp	r= -0.17 P= 0.20	r= -0.01 P= 0.93	r= -0.15 P= 0.26	r= 0.19 P= 0.15	r= -0.06 P= 0.67	r= 0.22 P= 0.09	r= -0.14 P= 0.27	**r= -0.34 P= 0.0090**		r= 0.26 P= 0.21	r= -0.12 P= 0.36
***VHL* inactivation**	r= 0.24 P= 0.23	**r= -0.49 P= 0.011**	r= -0.02 P= 0.92	r= 0.04 P= 0.83	r= -0.18 P= 0.36	r= -0.07 P= 0.71	r= 0.07 P= 0.71	r= 0.07 P= 0.74	r= 0.26 P= 0.21		**r= 0.42 P= 0.02**
***EGLN3* mRNA**	**r= 0.44 P= 0.00072**	r= -0.12 P= 0.36	r= -0.08 P= 0.53	r= -0.19 P= 0.15	r= -0.07 P= 0.60	r= -0.16 P= 0.22	r= 0.00 P= 0.98	**r= -0.46 P= 0.00019**	r= -0.12 P= 0.36	**r= 0.42 P= 0.022**	

Significant P values are shown in bold against a grey background.

ARTÍCULO 3: IL8 polymorphisms and overall survival in pazopanib- or sunitinib-treated patients with renal cell carcinoma.

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Resumen: En este estudio evaluamos la asociación de diferentes SNPs con la supervivencia global de pacientes con cáncer renal de células claras en estadio avanzado tratados tanto con sunitinib como con pazopanib.
Métodos: Inicialmente se determinaron 27 SNPs en 13 genes en una cohorte de descubrimiento consistentes en muestras de pacientes que habían participado en el ensayo fase III de registro de pazopanib en cáncer renal (N=241, estudio 1). Las asociaciones detectadas como significativas fueron validadas en dos cohortes adicionales: una procedente del ensayo denominado COMPARZ, fase III que comparó pazopanib frente a sunitnib (N=729, study 2) y el estudio observacional prospectivo SUT-REN 07 con pacientes tratados con sunitinib (N=89, study 3).
Resultados: En el estudio 1, se identificaron 4 polimorfismos con una asociación significativa (P≤0.05) con SG; dos de ellos (rs1126647, rs4073) en el gen IL8, también se asociaron con SG en el estudio 2 (P≤0.05). Dado que la correlación entre ambos SNPs fue muy alta, solo rs1126647 fue evaluado en el estudio 3, demostrando la misma tendencia de forma estadísticamente significativa (P≤0.05). Finalmente, un análisis combinado de los datos, demostró que el SNP rs1126647 se mantuvo asociado a SG, superando los ajustes para multiplicidad (P=8.8 × 10(-5); hazard ratio para la variante vs el alelo de referencia 1.32, (intervalo de confianza 95%: 1.15-1.52), sin evidencia de heterogeneidad de los efectos entre los diferentes estudios o entre el tratamiento con pazopanib y sunitinib.
Conclusiones: Los polimorfismos en el gen IL8 están asociados con una peor supervivencia y resultados en pacientes con cáncer renal de células claras tratados bien con sunitinib o pazopanib.

Aportación personal: Jesús García-Donas fue responsable de realizar los primeros contactos con los investigadores Xu Chun Fang y Tobey Johnson y proponer la realización de estudios conjuntos. Así mismo participó en las discusiones sobre el modo de cooperación y objetivos de la misma. Finalmente participó en la redacción y envío del manuscrito final.

FULL PAPER

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Keywords: pazopanib; sunitinib; renal cell carcinoma; IL8 polymorphism; overall survival; pharmacogenetics

IL8 polymorphisms and overall survival in pazopanib- or sunitinib-treated patients with renal cell carcinoma

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Background: We evaluated germline single nucleotide polymorphisms (SNPs) for association with overall survival (OS) in pazopanib- or sunitinib-treated patients with advanced renal cell carcinoma (aRCC).

Methods: The discovery analysis tested 27 SNPs within 13 genes from a phase III pazopanib trial ($N=241$, study 1). Suggestive associations were then pursued in two independent datasets: a phase III trial (COMPARZ) comparing pazopanib *vs* sunitinib ($N=729$, study 2) and an observational study of sunitinib-treated patients ($N=89$, study 3).

Results: In study 1, four SNPs showed nominally significant association ($P\leq0.05$) with OS; two of these SNPs (rs1126647, rs4073) in *IL8* were associated ($P\leq0.05$) with OS in study 2. Because rs1126647 and rs4073 were highly correlated, only rs1126647 was evaluated in study 3, which also showed association ($P\leq0.05$). In the combined data, rs1126647 was associated with OS after conservative multiple-test adjustment ($P=8.8\times10^{-5}$; variant *vs* reference allele hazard ratio 1.32, 95% confidence interval: 1.15–1.52), without evidence for heterogeneity of effects between studies or between pazopanib- and sunitinib-treated patients.

Conclusions: Variant alleles of *IL8* polymorphisms are associated with poorer survival outcomes in pazopanib- or sunitinib-treated patients with aRCC. These findings provide insight in aRCC prognosis and may advance our thinking in development of new therapies.

Renal cell carcinoma (RCC) is a heterogeneous collection of malignancies arising from the renal parenchyma, with >200 000 new cases worldwide per year (Gupta *et al*, 2008; Escudier *et al*, 2013). Although highly curable in its localised form by surgery, approximately a third of patients are diagnosed when metastatic spread has already occurred (Gupta *et al*, 2008); in addition,

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approximately 30% of patients surgically treated for a localised primary tumour will eventually develop metastases over time (Janowitz *et al*, 2013). The development of targeted systemic treatment options has improved clinical outcomes in patients with advanced and metastatic RCC (Molina and Motzer, 2011; Escudier *et al*, 2013; Fisher *et al*, 2013). Initial treatment decisions are based on prognosis of the disease using risk assessment models. Such prognostic models, initially developed for stratification of patients with metastatic RCC undergoing cytokine treatment (Motzer *et al*, 1999), have recently been extended to apply to patients receiving targeted therapies (Heng *et al*, 2009, 2013).

Pazopanib and sunitinib are angiogenesis inhibitors with highest affinity for vascular endothelial growth factor receptors, platelet-derived growth factor receptors, and stem cell factor receptor c-Kit (Motzer *et al*, 2007; Sternberg *et al*, 2010), and are approved for the treatment of advanced RCC. Treatment guidelines (Motzer *et al*, 2014; Escudier *et al*, 2014b) include both therapies as first-line options for RCC. Progression-free survival (PFS) benefits were observed for both sunitinib (*vs* interferon-alfa) and pazopanib (*vs* placebo) in their respective pivotal phase III studies (Motzer *et al*, 2007; Sternberg *et al*, 2010), but overall survival (OS) benefits were either marginal or not statistically significant (Motzer *et al*, 2007; Sternberg *et al*, 2013). However, these OS analyses were confounded by crossover or access to other therapies after progression. A recently completed phase III randomised clinical trial (COMPARZ) comparing pazopanib *vs* sunitinib for RCC demonstrated similar efficacies but differential safety profiles for the two therapies (Motzer *et al*, 2013), and a randomised cross-over phase III study (PISCES) demonstrated a significant patient preference for pazopanib over sunitinib with health-related quality of life and safety as key influencing factors (Escudier *et al*, 2014a).

There is substantial heterogeneity between patients with advanced RCC in prognosis and in response to treatment with targeted therapies, including pazopanib and sunitinib, and biomarkers that are predictive of clinical benefit would facilitate evidence-based selection of particular agents or dosages for optimal treatment of individual patients. The most obvious candidate biomarkers in clear-cell metastatic RCC (e.g., von Hippel–Lindau status, hypoxia-inducible factor (HIF) expression) have not been proven to have predictive significance (Fisher *et al*, 2013). As noted in recent reviews (Funakoshi *et al*, 2014; Maroto and Rini, 2014), some retrospective and prospective studies have reported potential molecular prognostic or predictive factors, including serum biomarkers (Tran *et al*, 2012; Harmon *et al*, 2014), tumour biomarkers (Rini *et al*, 2010; Dornbusch *et al*, 2013), and germline genetic variants (Garcia-Donas *et al*, 2011; van der Veldt *et al*, 2011; Xu *et al*, 2011; Scartozzi *et al*, 2013). Specifically, genetic polymorphisms in genes involved in sunitinib pharmacokinetics (e.g., CYP3A5, NR1I3, and ABCB1) or mode of action (e.g., VEGFR3) have recently been reported to be associated with PFS or OS in advanced RCC (Garcia-Donas *et al*, 2011; Scartozzi *et al*, 2013). We have previously reported that genetic polymorphisms in *IL8* and *HIF1A* may be associated with PFS, and polymorphisms in *HIF1A, NR1I2*, and *VEGFA* may be associated with best response (Xu *et al*, 2011). None of these genetic studies reported any attempt to confirm the association(s) in an independent dataset. To our knowledge, no prognostic or predictive biomarkers have yet to be prospectively validated in multiple independent studies to reliably distinguish patients with advanced RCC who are likely to respond from those who will not. To identify genetic predictors for OS, the present study used three independent datasets totalling 1059 pazopanib- or sunitinib-treated patients with advanced RCC.

MATERIALS AND METHODS

Patients. The discovery study (hereafter study 1) used data from participants in trials NCT00334282/VEG105192 and NCT00387764/VEG107769. The preplanned confirmation study (hereafter study 2) used data from participants in the COMPARZ trial: NCT00720941/VEG108844 and NCT01147822/VEG113078. Studies 1 and 2 included patients who provided written informed consent both for the clinical study and for genetic research. These clinical studies were conducted in accordance with the Declaration of Helsinki; protocols and informed consent forms were reviewed and approved by Institutional Review Boards and Independent Ethics Committees according to local guidelines. *Post hoc*, additional confirmation was sought using an observational study (hereafter study 3); the protocol was approved by the medical ethics review board of each participating institution and each participant provided written informed consent (Garcia-Donas *et al*, 2011).

Patient characteristics have been described previously (Sternberg *et al*, 2010; Garcia-Donas *et al*, 2011; Xu *et al*, 2011; Motzer *et al*, 2013) (Table 1). Briefly, NCT00334282 was a randomised, double-blind, placebo-controlled, pivotal phase III pazopanib study for advanced and/or metastatic RCC (Sternberg *et al*, 2010). NCT00387764 was an open-label extension to NCT00334282 providing the option for placebo-treated patients who developed progressive disease to receive pazopanib; OS for these patients was calculated from the time of initiation of pazopanib treatment in NCT00387764. Of the 369 patients enrolled in NCT00334282 and NCT00387764 who received pazopanib, study 1 used data from 241 patients who provided consent and a blood sample for genetic research. COMPARZ was a phase III randomised clinical trial comparing pazopanib *vs* sunitinib for metastatic RCC (Motzer *et al*, 2013). Of 1110 patients enrolled in COMPARZ, study 2 used data from 729 patients who received either pazopanib ($N=374$) or sunitinib ($N=355$) and provided consent and a blood sample for genetic research. Study 3 was an observational study undertaken by the Spanish Oncology GenitoUrinary Group (SOGUG); sunitinib-treated patients with clear-cell advanced RCC were included ($N=89$). Compared with the analysis reported previously by Garcia-Donas (27 patients died, median follow-up 21.2 months) (Garcia-Donas *et al*, 2011), this analysis uses data from extended follow-up for OS (50 patients died, median follow-up 36.9 months).

Procedures. For studies 1 and 2, germline DNA was extracted from peripheral blood (QiAamp DNA Blood Kit; Qiagen, Valencia, CA, USA). In the discovery analysis, 27 potential functional single nucleotide polymorphisms (SNPs) were selected from 13 candidate genes with evidence of involvement in angiogenesis or in the metabolism, disposition, or mode of action of pazopanib (Xu *et al*, 2011). Genotyping was conducted using single-base chain extension assays modified by GlaxoSmithKline (Research Triangle Park, NC, USA), TaqMan SNP assays (Applied Biosystems, Foster City, CA, USA), GoldenGate and Infinium genotyping assays (Illumina, San Diego, CA, USA), the KASPar SNP genotyping system (LGC Genomics, Hoddesdon, UK), and Sanger sequencing. For study 3, DNA was isolated from peripheral blood with FlexiGene DNA kit (Qiagen) or from saliva with Oragene DNA self-collection kits (DNA Genotek, Ottawa, Canada), and genotyping was conducted using the KASPar SNP genotyping system (Garcia-Donas *et al*, 2011).

All genotypes were called following the assay manufacturers' guidelines. Genotyping quality was confirmed by call rate, manual examination of cluster plots, concordance with previously reported allele frequencies, and checks of Hardy–Weinberg proportions

Table 1. Demographic and clinical characteristics of patients in the three studies

Characteristics	Discovery, Study 1 (pazopanib) *N* = 241	Confirmation, Study 2 (pazopanib or sunitinib) *N* = 729	Confirmation, Study 3 (sunitinib) *N* = 89
Age, median years (range)	60 (25–85)	61 (18–86)	65 (55–73)
Sex, *n* (%)			
Male	170 (71)	552 (76)	61 (69)
Race, self-reported, *n* (%)			
White	209 (87)	453 (62)	87 (98)
Non-white	32 (13)	276 (38)	2 (2)
Body mass index, median kg m^{-2} (range)	26 (14–46)	26 (15–55)	NA
ECOG PS or Karnofsky score, *n* (%)			
ECOG 0/KS 90–100	93 (39)	556 (76)	23 (28)
ECOG 1/KS 70–80	144 (60)	167 (23)	53 (64)
ECOG 2/KS ⩽60/missing[a]	4 (2)	6 (1)	13 (14)
MSKCC risk score, *n* (%)			
Favourable risk	94 (39)	211 (29)	27 (30)
Intermediate risk	130 (54)	420 (58)	44 (49)
Poor risk	2 (1)	73 (10)	2 (2)
Unknown	15 (6)	25 (3)	16 (18)
Time since initial diagnosis, *n* (%)			
⩽1 year	74 (31)	400 (55)	47 (53)
>1 year	139 (58)	329 (45)	42 (47)
Missing data	28 (12)	0	0
Prior nephrectomy status, *n* (%)			
Yes	216 (90)	611 (84)	76 (85)
No	23 (10)	118 (16)	13 (15)
Missing data	2 (<1)	0	0
LDH, *n* (%)			
⩽1·5 × ULN	215 (89)	673 (92)	82 (92)
>1·5 × ULN	16 (7)	45 (6)	4 (4)
Missing data	10 (4)	11 (2)	3 (3)
Haemoglobin, *n* (%)			
⩾LLN	129 (54)	445 (61)	55 (62)
<LLN	104 (43)	284 (39)	33 (37)
Missing data	8 (3)	0	1 (1)
Prior systemic treatment, *n* (%)			
Treatment-naive	128 (53)	729 (100)	89 (100)
Cytokine-pretreated	105 (44)	0	0
Missing data	8 (3)	0	0
Neutrophil count, *n* (%)			
⩽ULN	194 (81)	643 (88)	0
>ULN	39 (16)	82 (11)	0
Missing data	8 (3)	4 (1)	89 (100)
Platelet count, *n* (%)			
⩽ULN	184 (76)	629 (86)	0
>ULN	49 (20)	98 (13)	0
Missing data	8 (3)	2 (<1)	89 (100)
PFS, median weeks (95% CI)	38 (28–52)	48 (38–49)	55 (33–77)
OS, median months (95% CI)	25 (22–28)	32 (28–36)	27 (17–37)
Tumour objective response, n (%)[b]	83 (37)	241 (33)	39 (49)[c]

Abbreviations: CI = confidence interval; ECOG PS = Eastern Cooperative Oncology Group performance status; KS = Karnofsky score; LDH = lactate dehydrogenase; LLN = lower limit of normal range; MSKCC = Memorial Sloan-Kettering Cancer Center; OS = overall survival; PFS = progression-free survival; ULN = upper limit of normal range.
[a]One patient had missing baseline ECOG PS. All patients with ECOG PS of 2 or missing were from study VEG107769, had been randomised to the placebo arm of VEG105192, and later experienced disease progression while on treatment or during the follow-up period.
[b]Objective response represents complete response and partial response.
[c]Nine had missing data, 49% = 39 complete + partial responses/80 patients with data.

within self-reported non-Hispanic white patients and within self-reported East Asian patients.

Statistical analysis. In study 1 (discovery analysis), following Motzer *et al* (1999, 2002) and Heng *et al* (2009), baseline factors were first individually evaluated for association with OS using a univariate Cox proportional hazards model, and subsequently evaluated using multivariate stepwise model selection (forward selection at $P \leq 0.1$ to enter the model and backward selection at $P \leq 0.05$ to stay in the model) (Table 2). Each SNP was tested for association with OS using a multivariate Cox model, assuming an additive genetic model, and adjusting for the baseline factors identified by the stepwise model selection. SNPs showing nominal significance ($P \leq 0.05$ without adjustment for the number of SNPs tested) were considered for evaluation in study 2.

Table 2. Effect of baseline factors on overall survival in pazopanib-treated patients in the univariate and multivariate cox regression model in discovery study 1

	Univariate		Multivariate[a]	
Factors	**HR (95% CI)**	***P* value**	**HR (95% CI)**	***P* value**
Age, increase/year	1.00 (0.98–1.01)	0.9	—	—
Sex, female *vs* male	1.24 (0.88–1.73)	0.2	—	—
Race, self-reported other *vs* white	0.92 (0.57–1.48)	0.7	—	—
BMI, per $kg\,m^{-2}$	0.95 (0.91–0.98)	0.002	0.95 (0.92–0.99)	0.008
MSKCC risk score, intermediate/poor *vs* favourable	1.93 (1.37–2.73)	0.0002	—	—
ECOG PS, 1 or 2 *vs* 0[b]	1.73 (1.25–2.42)	0.001	1.63 (1.12–2.38)	0.01
Haemoglobin, $<$LLN *vs* $\geq$LLN[b]	1.53 (1.12–2.10)	0.008	—	—
LDH, $>1.5\times$ULN *vs* $\leq 1.5\times$ULN[b]	2.50 (1.41–4.42)	0.002	—	—
Prior nephrectomy status, no *vs* yes	1.33 (0.81–2.21)	0.3	—	—
Prior systemic treatment, treatment-naive *vs* cytokine-pretreated	1.24 (0.90–1.71)	0.2	—	—
Number of disease sites, ≥ 3 *vs* 1 or 2	1.24 (0.90–1.71)	0.003	1.56 (1.08–2.24)	0.02
Time from initial diagnosis to study entry, ≤ 1 year *vs* >1 year[b]	1.81 (1.29–2.54)	0.0006	1.50 (1.05–2.15)	0.03
Neutrophil count, ULN *vs* $\leq$ULN	1.82 (1.23–2.69)	0.003	1.66 (1.08–2.55)	0.02
Platelet count, $>$ULN *vs* $\leq$ULN	1.24 (0.86–1.80)	0.3	—	—
Study, VEG105192 *vs* VEG107769	1.06 (0.72–1.56)	0.8	—	—

Abbreviations: BMI = body mass index; CI = confidence interval; ECOG PS = Eastern Cooperative Oncology Group performance status; HR = hazard ratio; LDH = lactate dehydrogenase; LLN = lower limit of normal range; MSKCC = Memorial Sloan-Kettering Cancer Center; ULN = upper limit of normal range.
[a]For the multivariate model, HR and *P* values were shown for the final set of stepwise selected variables only; these variables were included as covariate(s) in the analysis of the effect of each genetic marker.
[b]These factors are also included in the calculation of the MSKCC risk score (Motzer *et al*, 1999, 2002).

In studies 2 and 3 (preplanned and *post hoc* confirmation analyses, respectively), SNPs were tested for association with OS using a multivariate Cox model, assuming an additive genetic model. In study 2, analyses were adjusted for the same baseline covariates as in study 1, plus ancestry principal components to adjust for confounding by population structure (Price *et al*, 2006). In study 3, analyses were adjusted for baseline Memorial Sloan-Kettering Cancer Center (MSKCC) risk score and sex, as described previously (Garcia-Donas *et al*, 2011). In parallel, SNPs nominally significantly associated ($P\leq 0.05$) with either PFS or best response, as reported previously (Xu *et al*, 2011), were also tested for association with either PFS or best response in study 2. All analyses in study 2 were conducted separately in pazopanib-treated and in sunitinib-treated patients, and also in a combined analysis of all patients (with additional covariate adjustment for treatment). Two-tailed *P* values were reported, and patients with missing baseline covariates or missing genotypes were excluded on a per-analysis basis.

Estimates of the natural-log hazard ratio (HR) from the multivariate Cox model analyses for study 1, from the two treatment arms of study 2 and from study 3, were combined using inverse variance weighted meta-analysis, assuming fixed effects and an additive genetic model. Heterogeneity of effects was assessed using the I^2 index of heterogeneity and by Cochran's Q statistic. For meta-analysis, a conservative significance threshold was determined using Bonferroni correction for all discovery-only and discovery-plus-confirmation analyses that could have been conducted (27 SNPs $\times 4$ sequential analyses with increasing cumulative sample sizes; threshold $0.05/27/4 = 4.6\times 10^{-4}$). Statistical analyses were conducted using the R system (Free Software Foundation, Boston, MA, USA), version 3.0.1.

RESULTS

We analyzed data from 1059 patients with advanced/metastatic RCC: 241 pazopanib-treated patients in study 1 (discovery); 374 pazopanib-treated and 355 sunitinib-treated patients in study 2 (preplanned confirmation); 89 sunitinib-treated patients in study 3 (*post hoc* confirmation). There was some heterogeneity in demographic and baseline clinical characteristics across the three datasets (Table 1). Similarly, there was a modest difference between studies in OS (Table 1); this likely reflects differences in baseline characteristics, as there was no significant difference in OS between pazopanib- and sunitinib-treated patients within study 2 (Motzer *et al*, 2013).

Table 2 lists the baseline factors evaluated in study 1 (discovery). Poor/intermediate MSKCC risk score, poor Eastern Cooperative Oncology Group (ECOG) performance status, low haemoglobin, high lactate dehydrogenase, high neutrophil count, increased number of disease sites, shorter time since initial diagnosis, and low body mass index were significantly associated with poor OS in univariate analyses. Multivariate stepwise model selection identified ECOG status, neutrophil count, number of disease sites, time since initial diagnosis, and body mass index as significantly associated with OS ($P\leq 0.05$, Table 2). In analyses adjusted for these five baseline factors, five of the 27 SNPs studied were associated with OS at $P\leq 0.05$ in the discovery study 1 (Table 3). Although no single association was significant after adjusting for the 27 SNPs studied, the number of associations at $P\leq 0.05$ was higher than expected by chance, and therefore we hypothesised that some of these associations might be confirmed in a larger independent dataset. Excluding the SNP where only two patients carried the variant genotype, four SNPs were analyzed further.

We hypothesised that in patients with advanced RCC, genetic effects on OS could be similar for angiogenesis inhibitors in the same class (i.e., pazopanib and sunitinib). Therefore, for follow-up of the discovery findings, we tested the four SNPs in both the pazopanib and sunitinib arms of study 2. In analysis of all patients in study 2, adjusted for treatment received, two *IL8* SNPs were associated with OS at $P\leq 0.05$ (rs1126647, Figure 1A; rs4073, Supplementary Figure S1). Genotypes at these two *IL8* SNPs were strongly correlated with each other (study 1 $r^2 = 0.79$; $P < 0.0001$),

Table 3. Association between genetic markers and overall survival in pazopanib-treated patients in discovery study 1

Gene	Polymorphism	rs Number	Minor Allele Frequency, %	*P* value[a]	HR[a] (95% CI)
CYP3A4	−392A>G	rs2740574	G 4.8	0.5	0.78 (0.39–1.55)
CYP3A5	6986A>G	rs776746	A 11.8	0.98	1.00 (0.68–1.45)
NR1I2	−25385C>T	rs3814055	T 39.0	0.02	1.34 (1.04–1.73)
NR1I2	7635A>G	rs6785049	G 40.6	0.7	0.96 (0.74–1.24)
NR1I2	10620C>T	rs1054190	T 9.0	0.7	1.11 (0.71–1.73)
ABCB1	1236C>T	rs1128503	T 43.2	0.054	1.25 (1.00–1.58)
ABCB1	2677G>T/A (A893S/T)	rs2032582	T/A 45.4	0.3	1.14 (0.89–1.47)
ABCB1	3435C>T	rs1045642	T 47.7	0.1	1.22 (0.94–1.58)
ABCG2	34G>A (V12M)	rs2231137	A 9.0	0.5	0.85 (0.55–1.33)
ABCG2	421C>A (Q141K)	rs2231142	A 13.5	0.7	1.06 (0.74–1.53)
ABCG2	869C>T (Q126X)	rs72552713	T 0.4	0.03[b]	4.81 (1.15–20.18)
VEGFA	−2578A>C	rs699947	C 47.9	0.2	0.85 (0.66–1.09)
VEGFA	−1498C>T	rs833061	T 47.7	0.2	0.85 (0.66–1.09)
VEGFA	−1154G>A	rs1570360	A 33.5	0.09	1.25 (0.96–1.63)
VEGFA	−634G>C	rs2010963	C 27.8	0.2	0.83 (0.62–1.11)
VEGFA	936C>T	rs3025039	T 15.3	0.4	1.15 (0.82–1.62)
VEGFR2	−604T>C	rs2071559	C 43.5	0.4	0.90 (0.70–1.14)
VEGFR2	889G>A (V297I)	rs2305948	A 8.9	0.4	1.20 (0.77–1.86)
VEGFR2	1416A>T (Q472H)	rs1870377	T 22.6	0.3	0.86 (0.64–1.16)
VEGFR3	1480A>G (T494A)	rs307826	G 8.6	0.2	1.36 (0.85–2.19)
PDGFRα	−573G>T	rs1800812	T 21.7	0.3	0.86 (0.66–1.13)
IL8	2767A>T	rs1126647	T 42.8	0.007	1.45 (1.11–1.91)
IL8	−251T>A	rs4073	A 49.8	0.02	1.36 (1.04–1.76)
FGF2	224C>T	rs1449683	T 8.8	0.3	1.29 (0.78–2.12)
FGFR2	IVS2 +906C>T	rs2981582	T 38.8	0.008	1.40 (1.09–1.81)
HIF1α	1772C>T (P582S)	rs11549465	T 10.3	0.6	0.88 (0.58–1.34)
HIF1α	1790G>A (A588T)	rs11549467	A 3.2	0.6	0.84 (0.42–1.65)

Abbreviations: CI = confidence interval; HR = hazard ratio.
[a]The HR and *P* values were from additive genetic models. The *P* values were nominal values without adjustment for the number of SNPs tested; HR values represent the per variant allele allelic HR.
[b]Although the ABCG2 rs72552713 was nominally significantly associated with overall survival, this association was driven by the only two patients with the variant CT genotype, and thus was not further discussed in this manuscript.

with rs1126647 (*IL8* 2767A>T) showing stronger association in both study 1 and study 2. Therefore, we focused on this SNP for a detailed description of the results. Given the inclusion of sunitinib-treated patients in study 2 but not in study 1, and a lack of other available genetic data for pazopanib-treated patients with advanced RCC, we sought additional confirmation for the association of rs1126647 in study 3.

In study 1 (pazopanib-treated, $N=241$), rs1126647 was significantly associated with OS ($N=186$; 125 events; $P=0.007$, per allele HR = 1.45, 95% confidence interval (CI): 1.11–1.91; 18 patients had missing genotype data and 37 patients had missing data for baseline factors) (Table 3, Figure 2). The effect size estimate was similar when baseline factors were not adjusted for ($N=223$; 148 events; $P=0.003$, per allele HR = 1.44, 95% CI: 1.13–1.83) (Figure 1A).

In study 2 (pazopanib- or sunitinib-treated, $N=729$), rs1126647 was significantly associated with OS ($N=690$; 287 events; $P=0.018$, HR = 1.23, 95% CI: 1.04–1.46, with adjustment for treatment; 10 patients had missing genotype data and 29 patients had missing data for baseline factors). The effect size estimate was similar when baseline factors were not adjusted for ($N=719$; 299 events; $P=0.014$, HR = 1.24, 95% CI: 1.04–1.46) (Figure 1B). The HRs for association between rs1126647 and OS were not significantly different between pazopanib-treated patients ($N=353$; 146 events; $P=0.53$, HR = 1.08, 95% CI: 0.84–1.40) and sunitinib-treated patients ($N=337$; 141 events; $P=0.008$, HR = 1.39, 95% CI: 1.09–1.77) in study 2 (Figure 2), with overlapping CIs (Figure 3) and no significant genotype by treatment interaction effect ($P=0.23$). The lack of a nominally significant association in pazopanib-treated patients in study 2 ($P=0.53$) precludes straightforward interpretation of these results, which is an issue we discuss further.

In study 3 (sunitinib-treated, $N=89$), a significant association between rs1126647 and OS, with similar effect size, was observed ($N=88$; 50 events; $P=0.034$, HR = 1.62, 95% CI: 1.04–2.54; one patient had missing genotype data) (Figures 1C and 2).

Meta-analysis of results from all three studies showed overall a significant association between *IL8* rs1126647 genotype and OS ($P=8.8\times10^{-5}$, HR = 1.32 per T allele, 95% CI: 1.15–1.52) (Figure 2) that was significant after Bonferroni correction for all discovery-only and discovery-plus-confirmation analyses that could have been conducted using the available data (threshold $P\leq4.6\times10^{-4}$). There was no significant heterogeneity in genetic effect size between studies ($I^2=19\%$, Cochran's Q = 3.73, 3 degrees of freedom, $P=0.29$).

Our previous pharmacogenetic analyses of pazopanib clinical trials for RCC (using data from study 1 plus an additional clinical trial that did not have OS data) suggested that three SNPs in the

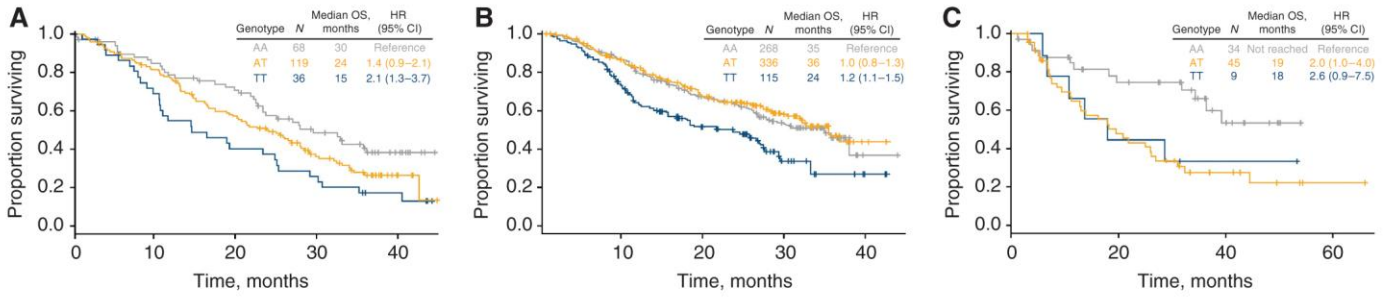

Figure 1. Overall survival Kaplan–Meier curves for patients by each *IL8* 2767A>T (rs1126647) genotype. (**A**) Pazopanib-treated patients in discovery study 1 (from NCT00334282 and NCT00387764): of the 241 patients, 223 had *IL8* genotype data and were included in this plot (including the 37 patients who had missing data for baseline factors). The remaining 18 patients had missing genotype data. (**B**) Pazopanib- or sunitinib-treated patients in confirmation study 2 (from COMPARZ): of the 729 patients, 719 had *IL8* genotype data and were included in this plot (including the 29 patients who had missing data for baseline factors). Ten patients had missing genotype data. (**C**) Sunitinib-treated patients in confirmation study 3 (SOGUG study): 88 of the 89 patients had *IL8* genotype data and were included in this plot; one patient had missing genotype data. The curves show the proportion of patients in each genotype group who survived (*y* axis) *vs* time in months (*x* axis). Vertical bars on the survival curves indicate censored observations. The HR was adjusted for covariates comparing each of the variant genotype (AT or TT) with the reference genotype (AA). AA, reference genotype; AT, variant heterozygote genotype; TT, variant homozygote genotype.

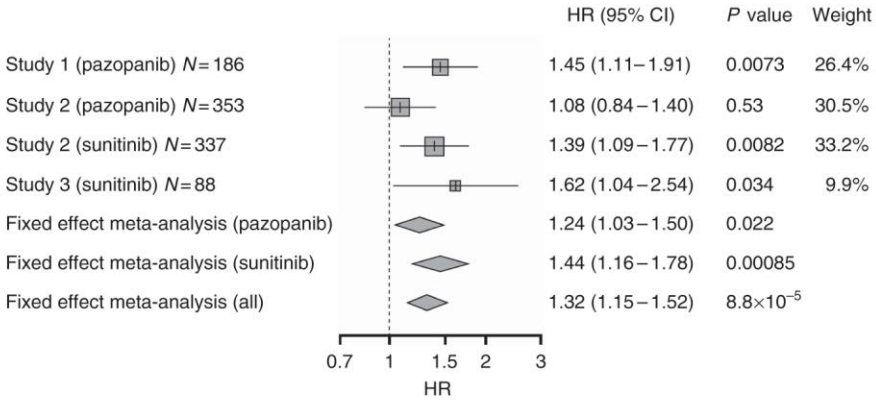

Figure 2. Forest plot of meta-analysis association results between *IL8* rs1126647 polymorphism and OS across three independent studies (with confirmation study 2 split into pazopanib- and sunitinib-treated subgroups). The HR was per variant T allele compared with reference A allele using an additive genetic model.

IL8 and *HIF1A* genes may be associated with PFS, and that five SNPs in the *HIF1A*, *NR1I2*, and *VEGFA* genes may be associated with best response (Xu *et al*, 2011). None of these SNPs showed nominally significant association (at $P \leq 0.05$) with either PFS or best response in follow-up analyses in the subset of pazopanib-treated patients from study 2, but *IL8* SNPs were weakly associated with PFS in sunitinib-treated patients.

DISCUSSION

Several antiangiogenesis agents are available for the treatment of advanced/metastatic RCC. However, few reliable predictors for treatment outcomes are available. The only externally validated models are the MSKCC or the International Metastatic Renal-Cell Carcinoma Database Consortium (IMDC) criteria that include baseline clinical factors to separate patients into risk categories with different prognoses (Motzer *et al*, 2002; Heng *et al*, 2013). Therefore, there is a growing interest in the field to explore pre-treatment demographic and clinical factors, serum/tissue biomarkers, and germline genetic markers that are potentially associated with efficacy endpoints. Here, using data from 1059 patients in three independent datasets, we report that rs1126647 in *IL8* is associated with OS in pazopanib- or sunitinib-treated patients with advanced RCC. Although the variant *IL8* genotype (TT) was associated with shorter OS than other genotypes, all genotype subgroups had survival benefit from treatment with pazopanib or sunitinib. For example, the median OS was 21.4–23.7 months for patients with the variant TT genotype and 27.8–35.5 months for the other genotypes in COMPARZ (Figure 3), all of which were substantially improved compared with historical survival data in advanced RCC when cytokines were the mainstay treatment (median survival 13 months) (Motzer *et al*, 2000). As the magnitudes of OS benefit vary depending on *IL8* genotypes, alternative sequencing or combination treatment strategies that are based on genotyping could be explored in the future as new therapies become available. Furthermore, the *IL8* genotype data could be incorporated into a prognostic model such as the existing IMDC model to improve the predictions of patients' clinical outcomes (Heng *et al*, 2013).

Progression-free survival has been used as a primary endpoint in some oncology clinical trials and has been considered an acceptable surrogate for OS in some settings (Shea *et al*, 2013; Bria *et al*, 2015). We were able to demonstrate association of *IL8* rs1126647 with OS but not with PFS. However, surrogacy with respect to the effect of a targeted therapy need not imply surrogacy with respect to effects of genetic differences, which may have distinct mechanism(s) of action (Fleming and DeMets, 1996). This raises the possibility that *IL8* variants may be associated with OS irrespective of treatment.

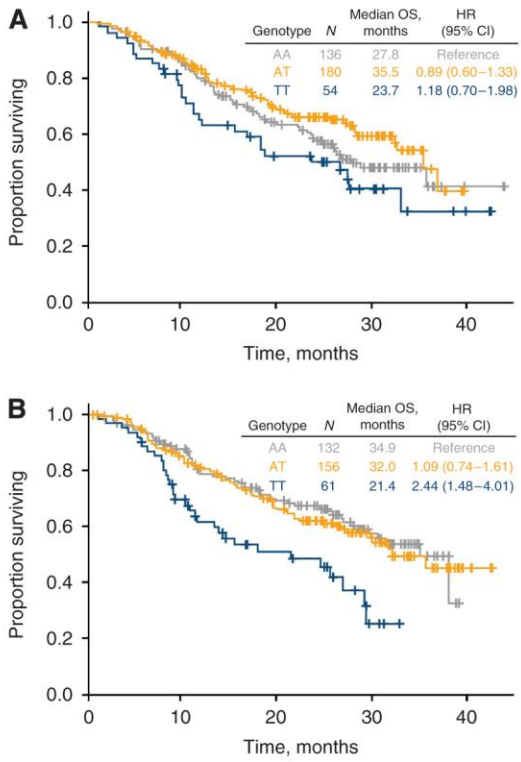

Figure 3. Overall survival (OS) Kaplan–Meier curves for *IL8* 2767A>T (rs1126647) genotype in confirmation study 2 (from COMPARZ) for (**A**) pazopanib-treated patients and (**B**) sunitinib-treated patients. Of the 729 patients, 719 had *IL8* genotype data and were included in this plot (including the 29 patients who had missing data for baseline factors). Ten patients had missing genotype data. AA, reference genotype; AT, variant heterozygote genotype; TT, variant homozygote genotype.

As with many cancer therapies, the benefit of antiangiogenic therapy is often transient in the metastatic disease setting, and there has been an ongoing search to identify mechanisms of resistance. Although clinical examples of clearly established mechanisms of resistance to angiogenesis inhibitors remain limited, findings from cell culture and murine model studies have revealed that activation of alternate or redundant signalling pathways may represent one such mechanism (Mizukami *et al*, 2005; Huang *et al*, 2010). The IL8 protein possesses mitogenic and angiogenic properties (Koch *et al*, 1992), and IL8-mediated angiogenesis was identified as a key compensatory mechanism of resistance to sunitinib in murine models of RCC (Huang *et al*, 2010). The *IL8* variant alleles evaluated in this study have been previously shown to be associated with increased gene expression (Hacking *et al*, 2004). Overexpression of IL8 is correlated with tumour stage, disease progression, and recurrence in various cancers (Yuan *et al*, 2005), as well as worse prognosis in localised RCC (Rini *et al*, 2010). In patients receiving pazopanib or sunitinib, high baseline serum IL8 levels were associated with shorter PFS and/or OS, suggesting that serum IL8 concentrations may be a prognostic or predictive factor for metastatic RCC (Liu *et al*, 2011; Tran *et al*, 2012; Harmon *et al*, 2014). One could therefore speculate that patients carrying the high-expression *IL8* variants may have more aggressive tumours and thus reduced survival *vs* those carrying the low-expression genotypes. It may be reasonable to consider IL8 blockade as a potential therapeutic target in future drug development for this patient subset.

Pharmacogenetic studies are often hampered by small sample sizes and limited availability of validation studies with eligibility criteria and treatment regimen similar to the discovery study. Strengths of the present study include a hypothesis-driven approach in a relatively large sample size study, the availability of one discovery and two confirmatory datasets, and detailed data on patient baseline characteristics. The prospective collection of germline DNA samples during pazopanib clinical trials enabled the evaluation of the effects of genetic markers on clinical response. Our evidence for association between OS and *IL8* rs1126647 in pazopanib- and sunitinib-treated patients with RCC is based on a combined analysis of all data available for this study. Clearly, these data were accumulated in stages, and the inclusion of study 3 data in our analysis was *post hoc*. Nonetheless, the strength of association based on all available data ($P = 8.8 \times 10^{-5}$) remains significant after a conservative multiple testing correction that accounts for the number of stages of data accumulation and also for the total number of SNPs that could have been followed through these stages (threshold $P \leq 4.6 \times 10^{-4}$). The evidence for association of *IL8* rs1126647 in patients treated with either pazopanib or sunitinib is supported by substantially overlapping 95% CIs from treatment-specific meta-analyses (pazopanib 95% CI: 1.03–1.50, sunitinib 95% CI: 1.16–1.78) (Figure 2). However, the association between OS and *IL8* rs1126647 in patients with RCC requires *bona fide* prospective validation in further independent studies.

In conclusion, data from the present study suggest that variant alleles (associated with high expression) in the *IL8* gene are associated with poorer survival outcome in patients with RCC who have received pazopanib or sunitinib. These findings provide additional scientific insight in the prognosis of advanced RCC after antiangiogenesis therapy, and may advance our thinking in developing new therapies.

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CONFLICT OF INTEREST

CFX, TJ, ZX, LM, JCW, CFS, and LNP are employees and stockholders of GlaxoSmithKline. NB and KCD were GlaxoSmithKline employees at the time of this study. JGD has received honoraria from GlaxoSmithKline, Pfizer, Novartis, and Bayer. CNS has received honoraria from GlaxoSmithKline, Pfizer, Novartis, and Bayer. IDD has been an advisory board member or chair for GlaxoSmithKline, Pfizer, Novartis, Janssen, Medivation, Sanofi, Bayer, Astellas, Ipsen, and Bristol-Myers Squibb, but all honoraria were directly donated to ANZUP Cancer Trials Group. TKC has received consultant compensation from GlaxoSmithKline, Pfizer, AVEO, Novartis, Genentech, Bayer, and Onyx. EE has been an advisory board member for GlaxoSmithKline, Pfizer, Novartis, and Bayer, but all honoraria were directly donated to UNDESO (foundation for the development of oncology research). RJM reports grants to his institution from GlaxoSmithKline, Pfizer, Novartis, and AVEO Oncology; he has received compensation from GlaxoSmithKline for travel and consultant compensation

from Pfizer and Genentech. CR-A declares that she has no conflicts of interest.

AUTHORS CONTRIBUTIONS

CFX, JG-D, ZX, and CR-A contributed to study design and conduct, acquisition of data, and data interpretation. TJ and NB contributed to study design, statistical analysis, and data interpretation. TKC, CNS, IDD, and RJM contributed to study design, acquisition of clinical data and DNA samples, and data interpretation. KCD, LM, and LNP contributed to study design and data interpretation. EE contributed to acquisition of data and data interpretation. JCW and CFS contributed to data interpretation. All authors contributed to the writing of this report and approved the final version.

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Supplementary Information accompanies this paper on British Journal of Cancer website (http://www.nature.com/bjc)

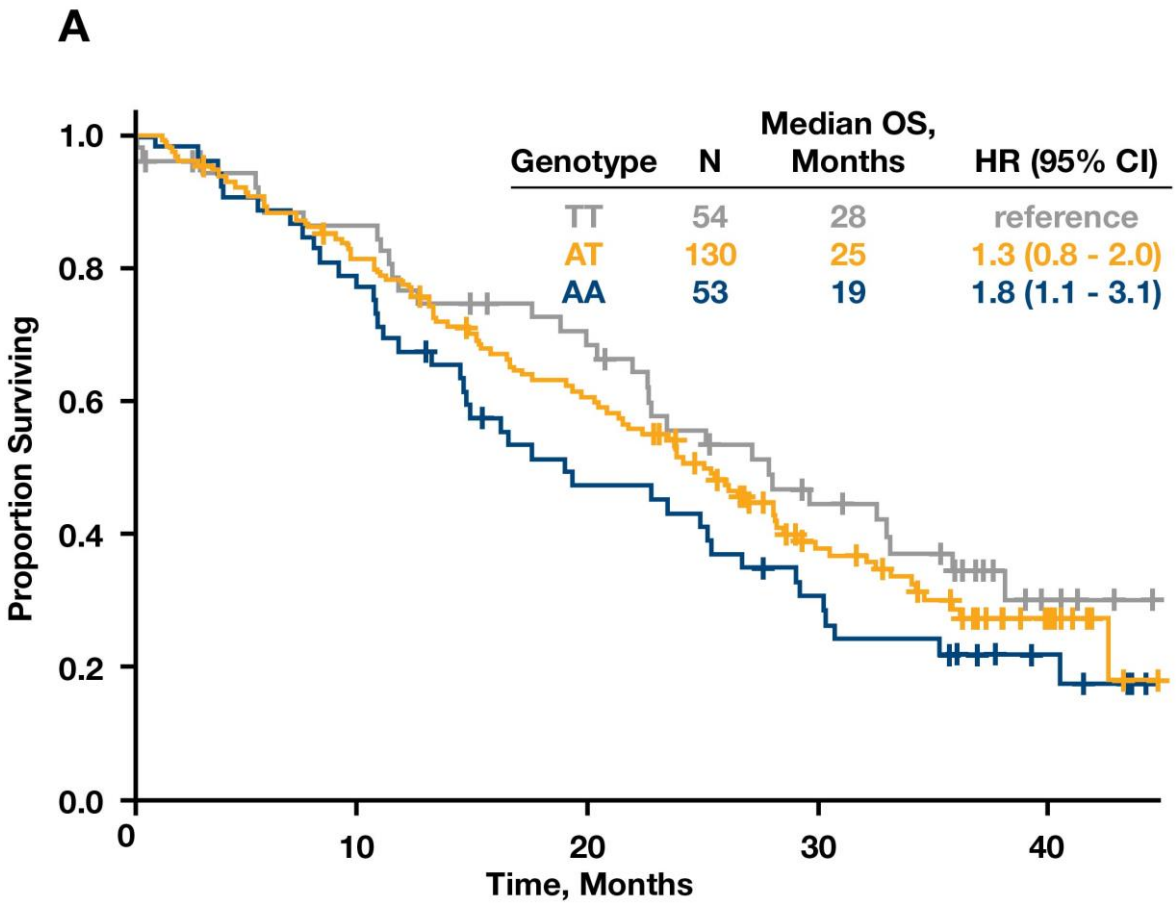
MATERIAL SUPPLEMENTARIO

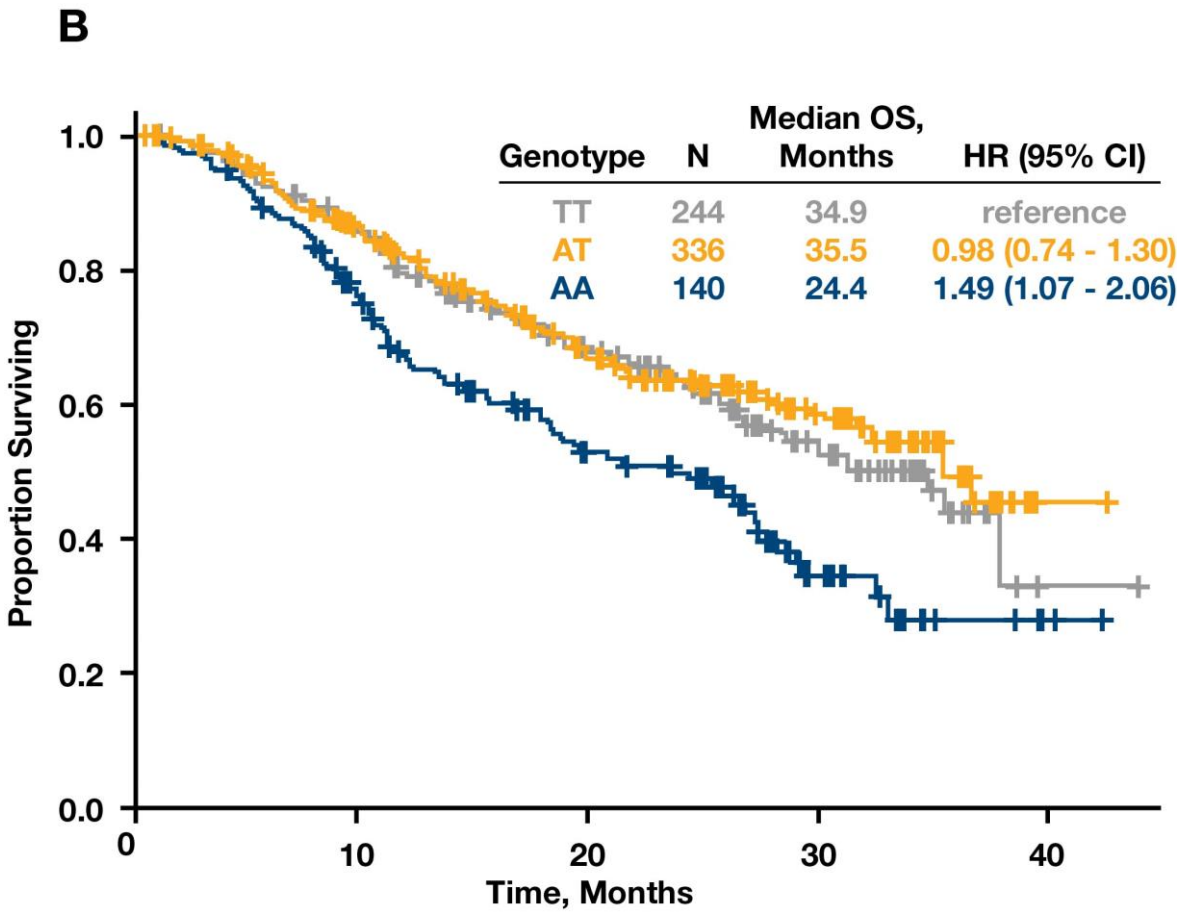
_IL8_ Polymorphisms and Overall Survival in Pazopanib- or Sunitinib-Treated Patients with Renal Cell Carcinoma

Xu C-F, Johnson T, Garcia-Donas J, et al

SUPPLEMENTARY MATERIAL

Supplementary Figure S1. Overall survival Kaplan-Meier curves without covariate for *IL8* -251T>A (rs4073) genotype in (A) discovery study 1 (pazopanib-treated patients) and (B) confirmation study 2 (pazopanib- and sunitinib-treated patients in COMPARZ). The alternate A allele at rs4073 (shown here) is the allele associated with the alternate T allele at rs1126647 (shown in Figure 1). AA, variant homozygote genotype; AT, variant heterozygote genotype; TT, reference genotype.

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Median OS,
Genotype N Months HR (95% CI)
TT 244 34.9 reference
AT 336 35.5 0.98 (0.74 - 1.30)
AA 140 24.4 1.49 (1.07 - 2.06)
Proportion Surviving
1.0
0.8
0.6
0.4
0.2
0.0
0
10
20
30
40
Time, Months

DISCUSIÓN

DISCUSIÓN

1- La población a estudio

La presente tesis doctoral se compone de tres trabajos distintos, y sus respectivas publicaciones, que fueron realizados sobre una misma cohorte de pacientes denominada SUT-REN-07.

Dicha cohorte consistió en la recogida sistemática y prospectiva de pacientes diagnosticados de cáncer renal de células claras en estadios avanzados que fuesen a ser tratados con sunitinib en primera línea dentro de la práctica asistencial.

Para ello, se contó con el soporte del grupo SOGUG que difundió la iniciativa entre todos sus socios, seleccionándose finalmente 15 centros. Debe destacarse que en el momento de la apertura del estudio sunitinib era la opción mayoritaria de tratamiento por no haberse aún aprobado pazopanib y ser muy escaso el empleo del esquema bevacizumab más interferón.

Se reclutó un total de 101 pacientes a lo largo de tres años consecutivos (2007 a 2010). Las características demográficas están detalladas en la tabla 1 del artículo 1 y son representativas del perfil de casos que suelen atenderse en esta patología.
Precisamente uno de los atractivos de este tipo de diseños, cohortes prospectivas dentro de la práctica asistencial, es su capacidad de reflejar la realidad de la consulta diaria con mayor precisión que los ensayo clínicos.

Así, no se excluyeron pacientes por comorbilidad ni otras circunstancias médicas y tan solo se exigió que los casos no hubieran recibido ningún tratamiento sistémico previo, ni siquiera citoquinas, y que el tumor primario presentara algún componente de células claras, sin especificar un porcentaje tumoral.

Estas mínimas restricciones pretendían evitar los sesgos derivados de la menor eficacia que los fármacos antiangiogénicos han demostrado después de una primera línea con citoquinas y la evolución dispar de las histologías no-células claras (lentas progresiones

en los casos de tumores cromófobos vs una extraordinaria agresividad en tumores sarcomatoides, por ejemplo).[50]

A pesar de no ser un estudio restrictivo en lo que a los criterios de selección se refiere, la tasa de reclutamiento fue lenta.
Esto probablemente refleja el momento histórico en que se realizó el estudio, ya que los fármacos antiangiogénicos habían sido aprobados de forma muy reciente.

Así, y aunque actualmente pueda resultar anacrónico, el cambio de paradigma que estas terapias representaron tardó varios años en consolidarse y no era infrecuente encontrar centros en los que pacientes con cáncer renal avanzado no eran remitidos a los servicios de oncología por los pobres resultados que habían ofrecido las terapias hasta entonces (basadas fundamentalmente en citoquinas).

Así, el uso de los antiangiogénicos, hoy claramente establecidos como referencia, tardó un tiempo en implantarse.

Además el trabajo fue uno de los primeros grandes estudios en biomarcadores del grupo SOGUG y pionero en abordar los mecanismo de resistencia a estas terapias en el mundo. Buena prueba de la novedad que supuso este trabajo ha sido la cantidad de subestudios que la cohorte SUT-REN 07 ha permitido (más de diez artículos diferentes publicados).

Gracias a esta población se han desarrollado colaboraciones a nivel europeo dentro del 7º Programa Marco en investigación de la Unión Europea (Proyecto EUROTARGET) y transcontinentales. De hecho, ocho años después de que se pusiera en marcha el estudio, nuestro grupo de investigación, dentro del SOGUG, sigue desarrollando trabajos adicionales con dos nuevos artículos redactados y pendientes de publicación.

En cierto modo, puede considerarse que el simple hecho de haber completado adecuadamente una recogida de casos y muestras biológicas de forma sistemática y con datos clínicos bien monitorizados, debe considerarse un éxito y una buena muestra de la visión de futuro de los investigadores que la hicieron posible.

2-La selección de las determinaciones

La aparición de los agentes antiangiogénicos supuso sin duda una revolución en la forma de manejar el cáncer renal.

El hecho de que se tratara de una patología particularmente compleja, por la ausencia de terapias útiles previas, hizo que el impacto fuera mayor que en otros tumores.

Curiosamente, le "elegancia" del racional biológico de estos tratamientos, despertó un cierto entusiasmo por desarrollar estudios que ahondaran en la biología molecular de la enfermedad y permitiese personalizar las terapias.

En nuestro caso, a diferencia de otros grupos, decidimos centrarnos en la investigación sobre polimorfismos por considerar que la principal diana de estos fármacos no sería la célula tumoral sino las células endoteliales.

Además, el estudio de polimorfismos presentaba la ventaja del fácil acceso a las muestras, dado que solo se requería una extracción de sangre periférica.

Uno de los problemas con los que nos enfrentamos al diseñar el trabajo inicial fue el número abrumador de polimorfismos descritos en la literatura. En aquel momento, no se disponía de las técnicas de Next Generation Sequencing (NGS) y los estudios con selecciones de SNPs más amplias empleaban mayoritariamente técnicas como el Genome Wide Association (GWAS) en la que se seleccionan en torno a un millón de variantes predeterminadas.

Dado que los estudios de GWAS requieren, por el número de variables que manejan, grandes tamaños muestrales, decidimos optar por una estrategia de "genes candidatos". Es decir, basándonos en la literatura, buscamos genes relacionados con las proteínas diana de los antiangiogénicos o con la metabolización de los fármacos. Además deberían presentar una "incidencia" (minor allele frequency) suficientemente alta como para esperar un número de casos relevante en nuestra población y que tuvieran significación clínica.

Con estos parámetros la Dra Rodriguez López de Antona seleccionó 16 candidatos, presentes en 9 genes.

3-Impacto de los SNPs en la respuesta al tratamiento y toxicidad con sunitinib

Como se detalla en el artículo 1, el análisis de la asociación de los SNPs elegidos y parámetros de eficacia arrojó varios resultados significativos, siendo lo más destacado la asociación de dos polimorfismos en el gen VEGFR3 (rs307826 y rs307821) con una peor supervivencia libre de progresión.

Esta asociación permaneció como significativa tras un análisis multivariante que incluyó las variables sexo y categoría pronóstica del MSKCC. Además, tras aplicar la corrección de Bonferroni para multiple testing, la "p" continuó siendo significativa.

Esta solidez en los resultados, es la mayor observada en ninguno de los diferentes trabajos llevados a cabo sobre SNPs en cáncer renal y respuesta a tratamiento y fue validada en un estudio independiente. [51]

De hecho, una de las líneas de investigación posteriores que se intentaron poner en marcha consistió en una propuesta de ensayo clínico fase II randomizado en el que los pacientes con cáncer renal metastásico serían genotipados para el SNP rs307826 y, en caso de resultar portadores, se aleatorizarían al tratamiento estandar (sunitinib/pazopanib) o a una alternativa, que en aquel momento se consideró que debería ser temsirolimus.

Esta iniciativa, denominada "estudio Genedrivity" (Genetically Driven Trial) contó con el apoyo de más de 20 centros distribuidos por 5 países de la UE y fue presentada a la última convocatoria de proyectos en salud del 7º programa Marco. Desgraciadamente, no obtuvo financiación por lo que no pudo llevarse a cabo.

Otro hallazgo relevante, fue la asociación del SNP CYP3A5 rs776746 con un mayor riesgo de toxicidad.

Este punto resultó especialmente complejo, dado que la gradación y causalidad de los

efectos secundarios, fuera de ensayo clínico, es una información especialmente difícil de extraer de las historias clínicas.

Por ello, decidimos seleccionar una serie de eventos que fueran "fácilmente identificables" y con una clara relación con la terapia (mucositis, síndrome palmo-plantar, hipertensión, anemia y trombocitopenia) o graves (cualquier toxicidad grado 3-4).

Aun así, dado que estos datos suelen estar pobremente recogidos en los evolutivos de la práctica diaria, establecimos una variable objetiva, el tiempo transcurrido desde el inicio de la terapia hasta la primera reducción de dosis, como la forma más fiable de determinar que había sucedido un evento clínicamente relevante.

Esta estrategia nos permitió determinar cómo el mencionado SNP se asoció con un mayor riesgo de reducción de dosis, superando igualmente las correcciones para multiplicidad.

Aunque se produjeron otros hallazgos interesantes, estos fueron menos robustos.

4-Impacto de la expresión de genes relacionados con la hipoxia en la respuesta a sunitinib y correlación entre los SNPs en VEGFR3 y expresión del gen

Desde el diseño del trabajo original, se consideró importante acompañar el estudio de SNPs en ADN germinal con la recogida de muestras tumorales de archivo.

Con esta estrategia se pretendía sacar el mayor rendimiento al importante esfuerzo logístico que representaba realizar un estudio multicéntrico a nivel nacional, de búsqueda de marcadores así como una forma de explorar el racional biológico de los hallazgos más relevantes que se produjeran en la determinación de los SNPs.

En línea con los estudios del momento, se exploraron algunas determinaciones como la presencia de alteraciones en el gen VHL que en varios trabajos se había apuntado como un posible determinante de eficacia.

Igualmente se incluyeron parámetros como la expresión del gen EGLN3 que en estudio previos de parte de los investigadores de nuestro grupo (Dras. Rodriguez Lopez de Antona y Robledo) habían demostrado ser relevantes en la patogenia del cáncer renal.

Aunque se comunicaron diversas asociaciones entre genes de hipoxia y respuesta a tratamiento, uno de los hallazgos más interesantes fue la correlación entre bajos niveles de expresión por inmunohistoquímica de la proteína VEGFR3 y la presencia del SNP rs 307826. De esta manera se sugería un potencial mecanismo por el que dicho SNP podía condicionar la respuesta a sunitinib. Así, una posible pérdida de estabilidad de la proteína potadora de la variante podría dar lugar a una expresión inferior y por tanto condicionaría una relativa resistencia.

Sin embargo, aunque interesante, no ha habido estudios que confirmen esta asociación ni que determinen el impacto biológico de la variante.

5-Estudio de validación externa en pacientes tratados con pazopanib

El articulo 3 presenta una estructura diferente a los otros dos por tratarse en este caso de una colaboración con un grupo externo que, al conocer los resultados iniciales de nuestra cohorte, nos contactó para validar los resultados que habían obtenido en sus propios pacientes.

El trabajo representa, por el número de casos estudiados, la mayor publicación sobre SNPs y predicción de respuesta a tratamiento en cáncer renal.

En este caso los investigadores, miembros de la empresa Glaxo Smithkline, trabajaron con muestras y datos clínicos procedentes de pacientes incluidos en dos ensayos con pazopanib que fueron claves en el desarrollo del fármaco.
El primero, denominado estudio 1 en el artículo, fue el ensayo de registro del medicamento en primera línea de cáncer renal y el segundo, el estudio COMPARZ, fue un fase III que enfrentó directamente pazopanib con sunitinib. [52]

El resultado final, la validación en tres cohortes distintas de un SNP en el gen de la interleukina 8 como predictor de peor respuesta al tratamiento antiangiogénico, es un hallazgo notorio.

Además, sabemos que la interleukina 8 es una proteína implicada en el proceso de la angiogénesis y se ha considerado como un potencial mecanismo de resistencia a inhibidores de VEGFR y VEGF. Por tanto, la plausibilidad biológica del hallazgo es clara.

6-Aplicación a la práctica asistencial

Aunque los estudios aquí presentados constituyen una sólida línea de trabajo que nos ha permitido desarrollar diferentes estudios en una de las patologías, el cáncer renal, de mayor interés para nuestro grupo de investigación, su aplicación a la práctica asistencial ha sido hasta el momento imposible.

Para ello se requeriría un ensayo clínico de validación que demostrara no solo el interés científico del papel de los SNPs en la resistencia a sunitinib, sino que confirmara que existe una alternativa terapéutica capaz de mejorar los resultados en esta población.

Aunque, como se ha mencionado, nuestro grupo llegó a diseñar dicho ensayo clínico (estudio Genedrivity) no pudo llevarse finalmente a acabo.

Es posible que la reciente aparición de terapias novedosas, como los checkpoint inhibitors, reabra el interés en este tipo de biomarcadores y permitan la implementación final del estudio de SNPs en la práctica clínica.

CONCLUSIONES

CONCLUSIONES

La predicción de eficacia y toxicidad a fármacos antiangiogénicos representa uno de los puntos clave para la personalización de las terapias en cáncer renal.
Esta necesidad será mas acuciante cuando los modernos fármacos inmunoterápicos se incorporen a la práctica asistencial, pues abrirá un nuevo abanico de posibilidades terapéuticas entre las que el clínico tendrá que elegir.

Nuestros estudios, centrados en el valor de los SNPs en este contexto, han demostrado que la colaboración multicéntrica y multidisciplinar permite completar estudios de calidad con resultados robustos que concuerdan con los resultados de series externas.

En concreto, hemos descubierto que existen dos polimorfismos en el gen VEGFR 3 (308266 y 308261) que se asocian a una peor respuesta a sunitinib. Además dichos SNPs se asocian a una menor expresión de la proteína, determinada por inmunohistoquímica, lo que explicaría el efecto biológico de la variante y el posible mecanismo de la resistencia.

Igualmente hemos detectado que un SNP (rs776746) en la ruta clave de metabolización de sunitinib, se asocia a un mayor riesgo de reducción de dosis. Esta asociación probablemente traduzca una mayor toxicidad por una exposición mayor a alguno de los metabolitos activos del fármaco, aunque este mecanismo no se ha estudiado adecuadamente.

Por último, trabajando con un grupo externo, hemos confirmado que un SNP en el gen IL8 (rs1126647) se asocia a una peor evolución. Dado que dicha proteína tiene una función bien conocida como estimulador de la angiogénesis, el hallazgo tiene una clara plausibilidad biológica.

Lamentablemente nuestros descubrimientos no podrán implementarse en la práctica asistencial por carecer de ensayos clínicos que avalen la utilidad de cambiar de terapia en base a la presencia de estos SNPs.

La consecución de este tipo de ensayos será una de las metas de nuestro grupo a medio plazo. Solo de esta manera conseguiremos extraer consecuencias prácticas para la asistencia médica a partir de los conocimientos moleculares.

APÉNDICE:
Otras publicaciones

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1-Nat Commun. 2015 Sep 25;6:8383.
A mutation in the POT1 gene is responsible for cardiac angiosarcoma in TP53-negative Li-Fraumeni-like families.

Calvete O, Martinez P, Garcia-Pavia P, Benitez-Buelga C, Paumard-Hernández B, Fernandez V, Dominguez F, Salas C, Romero-Laorden N, Garcia-Donas J, Carrillo J, Perona R, Triviño JC, Andrés R, Cano JM, Rivera B, Alonso-Pulpon L, Setien F, Esteller M, Rodriguez-Perales S, Bougeard G, Frebourg T, Urioste M, Blasco MA, Benítez J.

Cardiac angiosarcoma (CAS) is a rare malignant tumour whose genetic basis is unknown. Here we show, by whole-exome sequencing of a TP53-negative Li-Fraumeni-like (LFL) family including CAS cases, that a missense variant (p.R117C) in POT1 (protection of telomeres 1) gene is responsible for CAS. The same gene alteration is found in two other LFL families with CAS, supporting the causal effect of the identified mutation. We extend the analysis to TP53-negative LFL families with no CAS and find the same mutation in a breast AS family. The mutation is recently found once in 121,324 studied alleles in ExAC server but it is not described in any other database or found in 1,520 Spanish controls. In silico structural analysis suggests how the mutation disrupts POT1 structure. Functional and in vitro studies demonstrate that carriers of the mutation show reduced telomere-bound POT1 levels, abnormally long telomeres and increased telomere fragility.

2-Eur Urol. 2015 Oct;68(4):621-9.
CYP3A5 and ABCB1 Polymorphisms as Predictors for Sunitinib Outcome in Metastatic Renal Cell Carcinoma.

Diekstra MH, Swen JJ, Boven E, Castellano D, Gelderblom H, Mathijssen RH, Rodríguez-Antona C, García-Donas J, Rini BI, Guchelaar HJ.

BACKGROUND:
In our exploratory studies, we associated single nucleotide polymorphisms (SNPs) in candidate genes with the efficacy and toxicities of sunitinib in metastatic renal cell carcinoma (mRCC).
OBJECTIVE:
To see whether previously reported associations of SNPs with sunitinib-induced toxicities and efficacy in mRCC can be confirmed in a large cohort of patients.
DESIGN, SETTING, AND PARTICIPANTS:
The mRCC patients treated with sunitinib and a DNA sample available were pooled from three exploratory studies conducted in the United States, Spain, and the Netherlands. A total of 22 SNPs and 6 haplotypes in 10 candidate genes related to the pharmacokinetics and pharmacodynamics of sunitinib were selected for association testing.
OUTCOME MEASUREMENTS AND STATISTICAL ANALYSIS:
SNPs and haplotypes were tested for associations with toxicity, dose reductions, progression-free survival (PFS), overall survival (OS), and best objective response.
RESULTS AND LIMITATIONS:
A total of 333 patients were included. We confirmed 2 of the 22 previously reported SNP associations. The presence of CYP3A5*1 was associated with dose reductions (odds ratio: 2.0; 95% confidence interval [CI], 1.0-4.0, p=0.039). The presence of CGT in the ABCB1 haplotype was associated with an increased PFS (hazard ratio: 1.9; 95% CI, 1.3-2.6; p<0.001) and remained significant after Bonferroni correction. These associations are consistent with prior observations.
CONCLUSIONS:
The confirmation of previously reported associations between polymorphisms in CYP3A5 and ABCB1 with sunitinib toxicity and efficacy, respectively, indicates that genotyping of these genetic variants will be useful for guiding sunitinib treatment. A prospective validation study is needed to confirm our findings on ABCB1 and CYP3A5 genetic polymorphisms.
PATIENT SUMMARY:
We confirmed that variants in genes involved in processing sunitinib through the body have an effect on sunitinib treatment outcome. These findings confirm the potential of testing for these genetic variants to improve individual patient care for patients with

metastatic renal cell carcinoma treated with sunitinib.

3-Mol Oncol. 2015 Feb;9(2):422-36.
Deletion at 6q24.2-26 predicts longer survival of high-grade serous epithelial ovarian cancer patients.

Kamieniak MM, Rico D, Milne RL, Muñoz-Repeto I, Ibáñez K, Grillo MA, Domingo S, Borrego S, Cazorla A, García-Bueno JM, Hernando S, García-Donas J, Hernández-Agudo E, Y Cajal TR, Robles-Díaz L, Márquez-Rodas I, Cusidó M, Sáez R, Lacambra-Calvet C, Osorio A, Urioste M, Cigudosa JC, Paz-Ares L, Palacios J, Benítez J, García MJ.

Standard treatments for advanced high-grade serous ovarian carcinomas (HGSOCs) show significant side-effects and provide only short-term survival benefits due to disease recurrence. Thus, identification of novel prognostic and predictive biomarkers is urgently needed. We have used 42 paraffin-embedded HGSOCs, to evaluate the utility of DNA copy number alterations, as potential predictors of clinical outcome. Copy number-based unsupervised clustering stratified HGSOCs into two clusters of different immunohistopathological features and survival outcome (HR = 0.15, 95%CI = 0.03-0.81; Padj = 0.03). We found that loss at 6q24.2-26 was significantly associated with the cluster of longer survival independently from other confounding factors (HR = 0.06, 95%CI = 0.01-0.43, Padj = 0.005). The prognostic value of this deletion was validated in two independent series, one consisting of 36 HGSOCs analyzed by fluorescent in situ hybridization (P = 0.04) and another comprised of 411 HGSOCs from the Cancer Genome Atlas study (TCGA) (HR = 0.67, 95%CI = 0.48-0.93, Padj = 0.019). In addition, we confirmed the association of low expression of the genes from the region with longer survival in 799 HGSOCs (HR = 0.74, 95%CI = 0.61-0.90, log-rank P = 0.002) and 675 high-FIGO stage HGSOCs (HR = 0.76, 95%CI = 0.61-0.96, log-rank P = 0.02) available from the online tool KM-plotter. Finally, by integrating copy number, RNAseq and survival data of 296 HGSOCs from TCGA we propose a few candidate genes that can potentially explain the association. Altogether our findings indicate that the 6q24.2-26 deletion is an independent marker of favorable outcome in HGSOCs with potential clinical value as it can be analyzed by FISH on tumor sections and guide the

selection of patients towards more conservative therapeutic strategies in order to reduce side-effects and improve quality of life.

4-Clin Cancer Res. 2015 Jan 15;21(2):322-8.
Whole-exome sequencing reveals defective CYP3A4 variants predictive of paclitaxel dose-limiting neuropathy.

Apellániz-Ruiz M, Lee MY, Sánchez-Barroso L, Gutiérrez-Gutiérrez G, Calvo I, García-Estévez L, Sereno M, García-Donás J, Castelo B, Guerra E, Leandro-García LJ, Cascón A, Johansson I, Robledo M, Ingelman-Sundberg M, Rodríguez-Antona C.

PURPOSE:
Paclitaxel, a widely used chemotherapeutic drug, can cause peripheral neuropathies leading to dose reductions and treatment suspensions and decreasing the quality of life of patients. It has been suggested that genetic variants altering paclitaxel pharmacokinetics increase neuropathy risk, but the major causes of interindividual differences in susceptibility to paclitaxel toxicity remain unexplained. We carried out a whole-exome sequencing (WES) study to identify genetic susceptibility variants associated with paclitaxel neuropathy.
EXPERIMENTAL DESIGN:
Blood samples from 8 patients with severe paclitaxel-induced peripheral neuropathy were selected for WES. An independent cohort of 228 cancer patients with complete paclitaxel neuropathy data was used for variant screening by DHPLC and association analysis. HEK293 cells were used for heterologous expression and characterization of two novel CYP3A4 enzymes.
RESULTS:
WES revealed 2 patients with rare CYP3A4 variants, a premature stop codon (CYP3A4*20 allele) and a novel missense variant (CYP3A4*25, p.P389S) causing reduced enzyme expression. Screening for CYP3A4 variants in the independent cohort revealed three additional CYP3A4*20 carriers, and two patients with missense variants exhibiting diminished enzyme activity (CYP3A4*8 and the novel CYP3A4*27 allele, p.L475V). Relative to CYP3A4 wild-type patients, those carrying CYP3A4 defective

variants had more severe neuropathy (2- and 1.3-fold higher risk of neuropathy for loss-of-function and missense variants, respectively, P = 0.045) and higher probability of neuropathy-induced paclitaxel treatment modifications (7- and 3-fold higher risk for loss-of-function and missense variants, respectively, P = 5.9 × 10(-5)).
CONCLUSION:
This is the first description of a genetic marker associated with paclitaxel treatment modifications caused by neuropathy. CYP3A4 defective variants may provide a basis for paclitaxel treatment individualization.

5-PLoS One. 2014 Jan 24;9(1):e86263.
Identification of tissue microRNAs predictive of sunitinib activity in patients with metastatic renal cell carcinoma.

Prior C, Perez-Gracia JL, Garcia-Donas J, Rodriguez-Antona C, Guruceaga E, Esteban E, Suarez C, Castellano D, del Alba AG, Lozano MD, Carles J, Climent MA, Arranz JA, Gallardo E, Puente J, Bellmunt J, Gurpide A, Lopez-Picazo JM, Hernandez AG, Mellado B, Martínez E, Moreno F, Font A, Calvo A.

PURPOSE:
To identify tissue microRNAs predictive of sunitinib activity in patients with metastatic renal-cell-carcinoma (MRCC) and to evaluate in vitro their mechanism of action in sunitinib resistance.
METHODS:
We screened 673 microRNAs using TaqMan Low-density-Arrays (TLDAs) in tumors from MRCC patients with extreme phenotypes of marked efficacy and resistance to sunitinib, selected from an identification cohort (n = 41). The most relevant differentially expressed microRNAs were selected using bioinformatics-based target prediction analysis and quantified by qRT-PCR in tumors from patients presenting similar phenotypes selected from an independent cohort (n = 101). In vitro experiments were conducted to study the role of miR-942 in sunitinib resistance.
RESULTS:
TLDAs identified 64 microRNAs differentially expressed in the identification cohort. Seven candidates were quantified by qRT-PCR in the independent series. MiR-942 was

the most accurate predictor of sunitinib efficacy (p = 0.0074). High expression of miR-942, miR-628-5p, miR-133a, and miR-484 was significantly associated with decreased time to progression and overall survival. These microRNAs were also overexpressed in the sunitinib resistant cell line Caki-2 in comparison with the sensitive cell line. MiR-942 overexpression in Caki-2 up-regulates MMP-9 and VEGF secretion which, in turn, promote HBMEC endothelial migration and sunitinib resistance.
CONCLUSIONS:
We identified differentially expressed microRNAs in MRCC patients presenting marked sensitivity or resistance to sunitinib. MiR-942 was the best predictor of efficacy. We describe a novel paracrine mechanism through which high miR-942 levels in MRCC cells up-regulates MMP-9 and VEGF secretion to enhance endothelial migration and sunitinib resistance. Our results support further validation of these miRNA in clinical confirmatory studies.

6-Br J Cancer. 2013 Apr 30;108(8):1732-42.
DNA copy number profiling reveals extensive genomic loss in hereditary BRCA1 and BRCA2 ovarian carcinomas.

Kamieniak MM, Muñoz-Repeto I, Rico D, Osorio A, Urioste M, García-Donas J, Hernando S, Robles-Díaz L, Ramón Y Cajal T, Cazorla A, Sáez R, García-Bueno JM, Domingo S, Borrego S, Palacios J, van de Wiel MA, Ylstra B, Benítez J, García MJ.

BACKGROUND:
Few studies have attempted to characterise genomic changes occurring in hereditary epithelial ovarian carcinomas (EOCs) and inconsistent results have been obtained. Given the relevance of DNA copy number alterations in ovarian oncogenesis and growing clinical implications of the BRCA-gene status, we aimed to characterise the genomic profiles of hereditary and sporadic ovarian tumours.
METHODS:
High-resolution array Comparative Genomic Hybridisation profiling of 53 familial (21 BRCA1, 6 BRCA2 and 26 non-BRCA1/2) and 15 sporadic tumours in combination with supervised and unsupervised analysis was used to define common and/or specific

copy number features.
RESULTS:
Unsupervised hierarchical clustering did not stratify tumours according to their familial or sporadic condition or to their BRCA1/2 mutation status. Common recurrent changes, spanning genes potentially fundamental for ovarian carcinogenesis, regardless of BRCA mutations, and several candidate subtype-specific events were defined. Despite similarities, greater contribution of losses was revealed to be a hallmark of BRCA1 and BRCA2 tumours.
CONCLUSION:
Somatic alterations occurring in the development of familial EOCs do not differ substantially from the ones occurring in sporadic carcinomas. However, some specific features like extensive genomic loss observed in BRCA1/2 tumours may be of clinical relevance helping to identify BRCA-related patients likely to respond to PARP inhibitors.

7-Pharmacogenomics J. 2011 Apr;11(2):121-9.
Polymorphisms in cytochromes P450 2C8 and 3A5 are associated with paclitaxel neurotoxicity.

Leskelä S1, Jara C, Leandro-García LJ, Martínez A, García-Donas J, Hernando S, Hurtado A, Vicario JC, Montero-Conde C, Landa I, López-Jiménez E, Cascón A, Milne RL, Robledo M, Rodríguez-Antona C.

Neurotoxicity is one of the most relevant dose-limiting toxicities of the anticancer drug paclitaxel. It exhibits substantial interindividual variability of unknown molecular basis, and represents one of the major challenges for the improvement of paclitaxel therapy. The extensive variability in paclitaxel clearance and metabolism lead us to investigate the association between polymorphisms in paclitaxel elimination pathway and neurotoxicity. We selected 13 relevant polymorphisms in genes encoding paclitaxel metabolizing enzymes (CYP2C8, CYP3A4 and CYP3A5) and transporters (organic anion transporting polypeptide (OATP) 1B1, OATP1B3 and P-glycoprotein) and genotyped them in 118 Spanish cancer patients treated with paclitaxel. After adjusting for age and treatment schedule, CYP2C8 Haplotype C and CYP3A5*3 were associated

with protection (hazard ratio (HR) (per allele)=0.55; 95% confidence interval (CI)=0.34-0.89; P=0.014 and HR (per allele)=0.51; 95%CI=0.30-0.86; and P=0.012, respectively) and CYP2C8*3 with increased risk (HR (per allele)=1.72; 95%CI=1.05-2.82; and P=0.032). In each case, the allele causing increased paclitaxel metabolism was associated with increased neurotoxicity, suggesting an important role for metabolism and hydroxylated paclitaxel metabolites. We estimated the HR per paclitaxel-metabolism increasing allele carried across the three polymorphisms to be HR=1.64 (95% CI=1.26-2.14; P=0.0003). The results for P-glycoprotein were inconclusive, and no associations were observed for the other genes studied. The incorporation of this genetic data in treatment selection could help to reduce neurotoxicity events, thereby individualizing paclitaxel pharmacotherapy. These results warrant validation in independent series.

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